

Interactions Between Rheumatoid Factor IgM and Immune Complexes, and Conformational Anomalies in Immunoglobulins from Rheumatoid and Hyperviscous Sera

Ulf Alkner



Lund 1983

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and Conformational Anomalies in Immunoglobulins from Rheumatoid
and Hyperviscous Sera

by

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Abstract RF IgM was shown to be capable of dissociating soluble antigen-antibody complexes formed in gross antigen excess. This new effect attributed to RF was due to the presence of low affinity RF. The dissociation occurred irrespective of the type of antigen and the origin of the antibody. Normal IgM was shown to have a previously unknown ability to enhance the precipitation of soluble immune complexes formed in gross antigen excess, the effect being probably due to a hydrophobic interaction between normal IgM and the immune complexes. Intermediate complexes (IC;IgG-IgG complexes) isolated from rheumatoid or hyperviscous sera (or sera that were both rheumatoid and hyperviscous) were found to contain IgG with unique antigenic determinants not found in normal IgG, and unrelated to RF activity. In one case they were located to the F(ab')₂γ part. The IgG in IC also exhibited a subclass restriction and contained an abnormally high amount of sialic acids. Circular dichroism analysis of IgA isolated from the serum of a patient with hyperviscosity syndrome revealed differences between the IgA in complexes and the 7S IgA. The deviations were related to differences in the exposure of aromatic amino acids, and possibly in the micro-environment of disulphide bridges. The same principal deviations as compared to normal IgG, were also found in the IgG with the unique determinants, isolated from a patient with the hyperviscosity syndrome. Furthermore, the hyperviscous IgA was found to be more hydrophobic than normal IgA.		
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Date April 8, 1983

**Interactions Between
Rheumatoid Factor IgM and Immune Complexes,
and Conformational Anomalies in Immunoglobulins
from Rheumatoid and Hyperviscous Sera**



Lund 1983

Interaction Between
Phenothiazine Receptor Ligand and Immune Components
and Cardiovascular Alterations in Hypertensive Rats
from Phenothiazine and Hypertensive Rats

LUND 1983
BLOMS BOKTRYCKERI AB

A description of the rheumatoid factor or

"..... a bad man who does good things or a good man
who does bad things."

(Somerset Maugham)

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PUBLICATIONS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I. Alkner, U., and Hansson, U.-B. Effect of IgM fractions with or without rheumatoid factor activity on antigen-antibody reactions. *Scand. J. Immunol.* 10, 119, 1979.
- II. Alkner, U. Dissociation of soluble antigen-antibody complexes by rheumatoid factor IgM. Submitted for publication in *Scand. J. Immunol.*
- III. Hansson, U.-B., Uesson, M., and Alkner, U. Isolation and characterization of intermediate complexes and other components with common antigenic determinants. *Scand. J. Immunol.* 13, 56, 1981.
- IV. Alkner, U., Uesson, M., Hansson, U.-B., and Glans, J. Circular dichroism of immune complexes with rheumatoid factor activity. *Molecular Immunology* 19, 21, 1982.
- V. Alkner, U., Hansson, U.-B., and Lindström, F.D. Factors affecting IgA related hyperviscosity. *Clin. exp. Immunol.* 51, 617, 1983.

PURPOSE OF THE STUDY

Rheumatoid factors (RF) and circulating immune complexes are serological features characteristic of rheumatoid arthritis. Both positive and negative biological properties have been attributed to RF, especially when its reaction with immune complexes, with immunoglobulin complexes, or with complement are discussed. Circulating immune complexes and immunoglobulin complexes are also often found to occur in the serum from individuals with hyperviscosity syndrome and may well be related to the disease.

The mechanisms behind the reactions between RF and immune complexes, the mechanisms for the formation of circulating immune complexes in rheumatoid arthritis, and the structural characteristics of the molecules which are related to the development of rheumatoid arthritis and to the hyperviscosity syndrome, are all of importance both from a biochemical and medical point of view.

The purpose of this study was to ascertain the effects of RF IgM and normal IgM on soluble and immobilized immune complexes, to characterize immune complexes and immunoglobulin complexes in rheumatoid and hyperviscous sera, and to determine eventual conformational deviations of the immunoglobulins in these complexes.

INTRODUCTION

The human body has a highly organized defence system against invading organisms and "foreign" molecules (antigens). The immune system is vital and includes various organs such as the thymus, spleen, lymph nodes and bone marrow, and several cell types such as lymphocytes, plasma cells, macrophages and granulocytes. The function of the immune system is to neutralize and eliminate "foreign" matter, the two main ways this is accomplished being cell mediated immunity - with macrophages and proliferated T-cells as two of the mediators, and humoral immunity - with antibodies as the principal mediators. This thesis deals with one facet of humoral immunity, and is focused on the reaction between the rheumatoid factor and antigen-antibody complexes, and on the immunoglobulin complexes found in serum from patients with rheumatoid arthritis or hyperviscosity syndrome or both.

Immunoglobulin structure

According to the clonal selection theory, antibodies are produced by B-lymphocytes, carrying on their surfaces pre-produced receptor antibodies directed against a specific antigen. The antigen selects its own clone of cells, binds to the receptor, and induces a proliferation resulting in an enhanced antibody production. According to the somatic mutation theory, the diversity of the immune system is due to a random combination of gene segments; theoretically, $10^7 - 10^9$ different antibody molecules, each with different specificities, can be produced.

The immunoglobulins, the antibodies, are globular glycoproteins with a basic four-chain structure (Fig. 1). They are composed of two identical heavy polypeptide chains ($M = 51000 - 76000$), and two identical light polypeptide chains ($M = 22000 - 23000$). One light chain and one heavy chain are combined via disulphide bridges into a half-molecule which binds, via disulphide bridges between the heavy chains, to another identical half molecule. Each polypeptide chain is composed of globular domains, and loosely folded switch regions between them, the domains being stabilized by intrachain disulphide bridges. The light

chain has two domains and the heavy chain four to five, each domain (~110 amino acid residues) having a hydrophobic core and high amount of β -structure with random structures between the sheets. The amino acid sequence of the polypeptide chains varies substantially in the amino terminal parts. Within this segment are situated the hyper-variable regions which are brought together by the folding of the polypeptide chain. The variable light (V_L) chain and variable heavy chain (V_H) domains form the antigen binding sites (Fig. 1). The amino acid sequence within the other domains, the C_H and C_L domains, is more constant in a given class or subclass (see below). These domains are responsible for many biological effects and also contain the bulk of the carbohydrates (Fig. 1 - 3). Carbohydrates have, however, also been found in the variable part of the immunoglobulins (Matsuuchi et al., 1981; Sox and Hood, 1970; Spiegelberg et al., 1970). The carbohydrate is made up of branched oligosaccharides containing fucose, galactose, mannose, N-acetyl-glucosamine, N-acetyl-galactosamine and neuraminic acid in various combinations, depending on the immunoglobulin class. If present, neuraminic acid is situated at the outer end of the oligosaccharides. The role of the carbohydrates in immunoglobulins is not clearly understood, but they stabilize the structure of the molecule and perhaps contribute to the secretion of the immunoglobulins (Nisonoff et al., 1975), and their subsequent elimination (Finbloom et al., 1981; Morell et al., 1971; Rifai et al., 1982).

There are two types of light chains, kappa (κ) and lambda (λ), and five types of heavy chains. The amino acid sequence of the constant part of the heavy chains denotes which of the five immunoglobulin classes (heavy chains) are present - IgA(α), IgD(δ), IgE(ϵ), IgG(γ) or IgM(μ). Each antibody molecule has one type of light chain and one type of heavy chain.

Both the variable and the constant regions have certain restricted areas. The variable regions can thus be divided into three families, the kappa, lambda and heavy chain families, implying that more than one type of heavy chain may share one variable region. Idiotypic determinants are certain molecular structures located in the variable

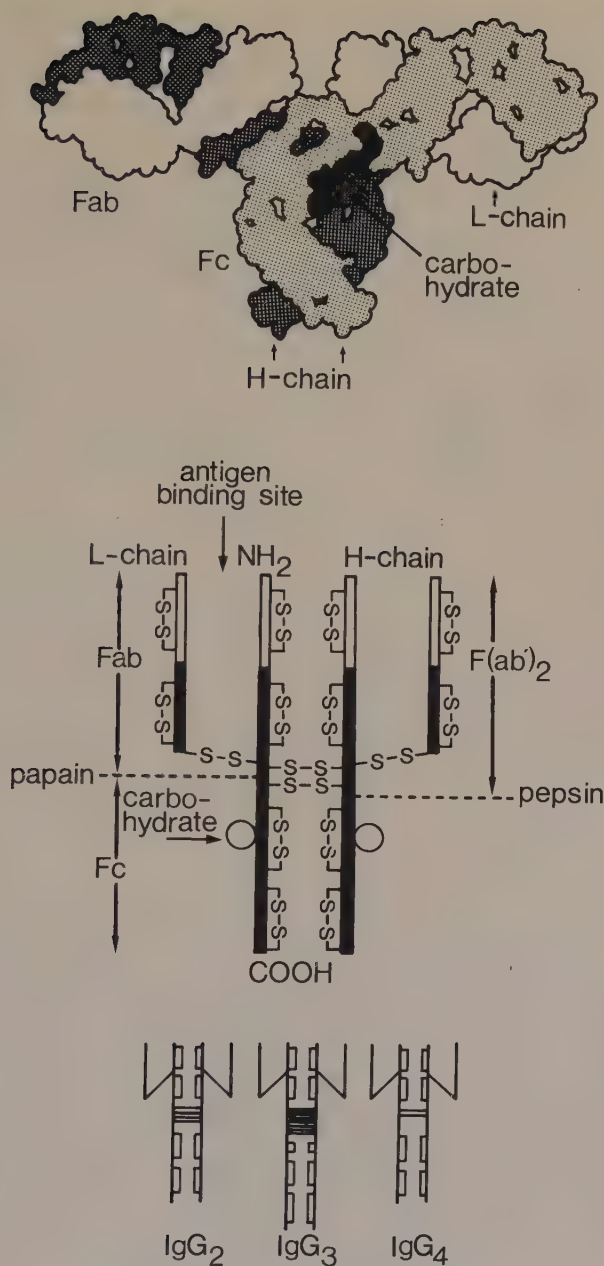


Figure 1: Top: Space filling model of an IgG molecule (After Silvertown, E.W. et al., Proc. Natl. Acad. Sci. USA 74, 5140, 1977).

Centre: The four chain structure of IgG 1.

Bottom: The arrangement of the disulphide bridges in IgG2, 3 and 4.

regions and related to antibody activity. Depending upon what amino acid residues are present in the restricted areas within the constant parts, the kappa light chains can be divided into three subtypes, and the heavy chain classes into subclasses. The constant part of the kappa light chain, and of the α , γ and μ chains, contain certain genetic markers, the allotypes. These markers are inherited amino acid substitutions which comprise one or more amino acid residues.

IgG ($M = 150000$) has four subclasses differing in number and location of their interchain disulphide bridges (Fig. 1). They also differ in certain biological activities - for example, in their ability to fix complement and to bind to cell membranes.

IgM ($M = 970000$) has a pentameric structure where each subunit has the basic four chain structure (Fig. 2). The μ chain has four constant domains compared to three for the γ chain and the α chain (Fig. 1 - 3). The subunits are linked together by disulphide bridges and a special polypeptide called the J-chain. The central plate, $(Fc)_5\mu$, is a rigid structure, and due to the compact and cyclical structure of the IgM molecule, movement of the Fab arms is restricted compared with that of the IgG molecule.

IgA mainly occurs in serum as monomer ($M = 150000$) (Fig. 3), though 10 - 15 % of the IgA in normal human serum may occur as dimers or polymers (Tomasi and Grey, 1972). The IgA in complexes are often covalently linked by disulphide bridges and a J-chain (Fig. 3). IgA has two subclasses, IgA1 and IgA2; IgA2 differs from the four chain units of the other immunoglobulins, its light chains being joined by a disulphide bridge and bound to the heavy chains by non-covalent forces (Fig. 3).

The J-chain ($M = 15000$) is a very hydrophilic polypeptide with very few aromatic amino acid residues, and possibly no tryptophane (Cunningham-Rundles, 1978; Koshland, 1975). It contains some carbohydrate and is disulphide linked to IgM or IgA dimers and polymers.

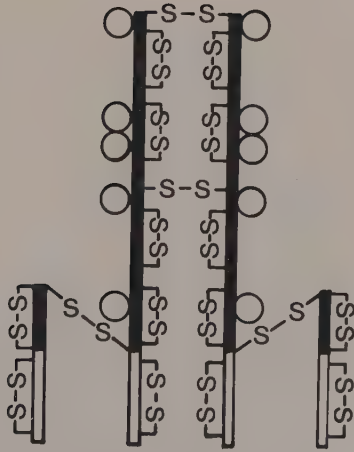
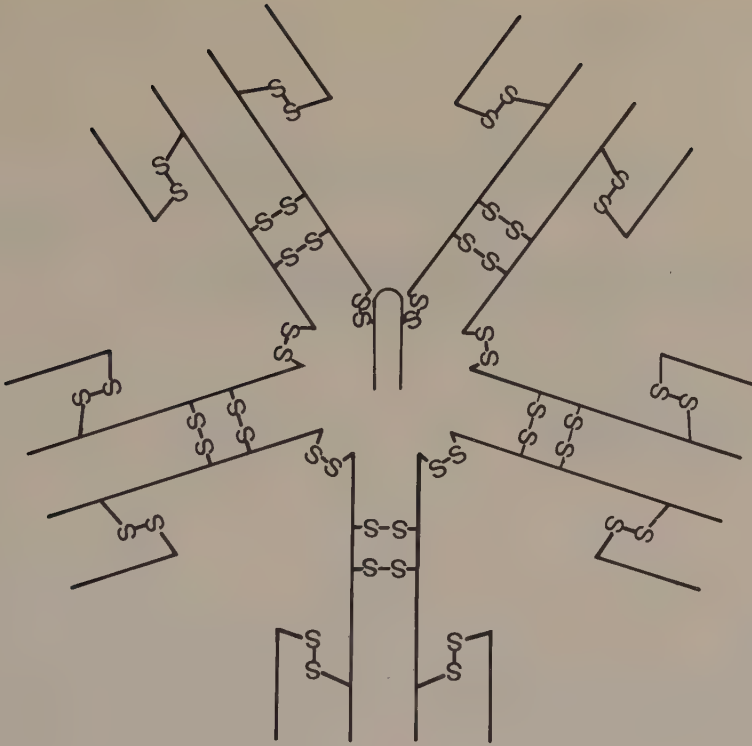


Figure 2: Top: The pentameric structure of IgM.
 Bottom: The four chain structure of an IgM subunit.

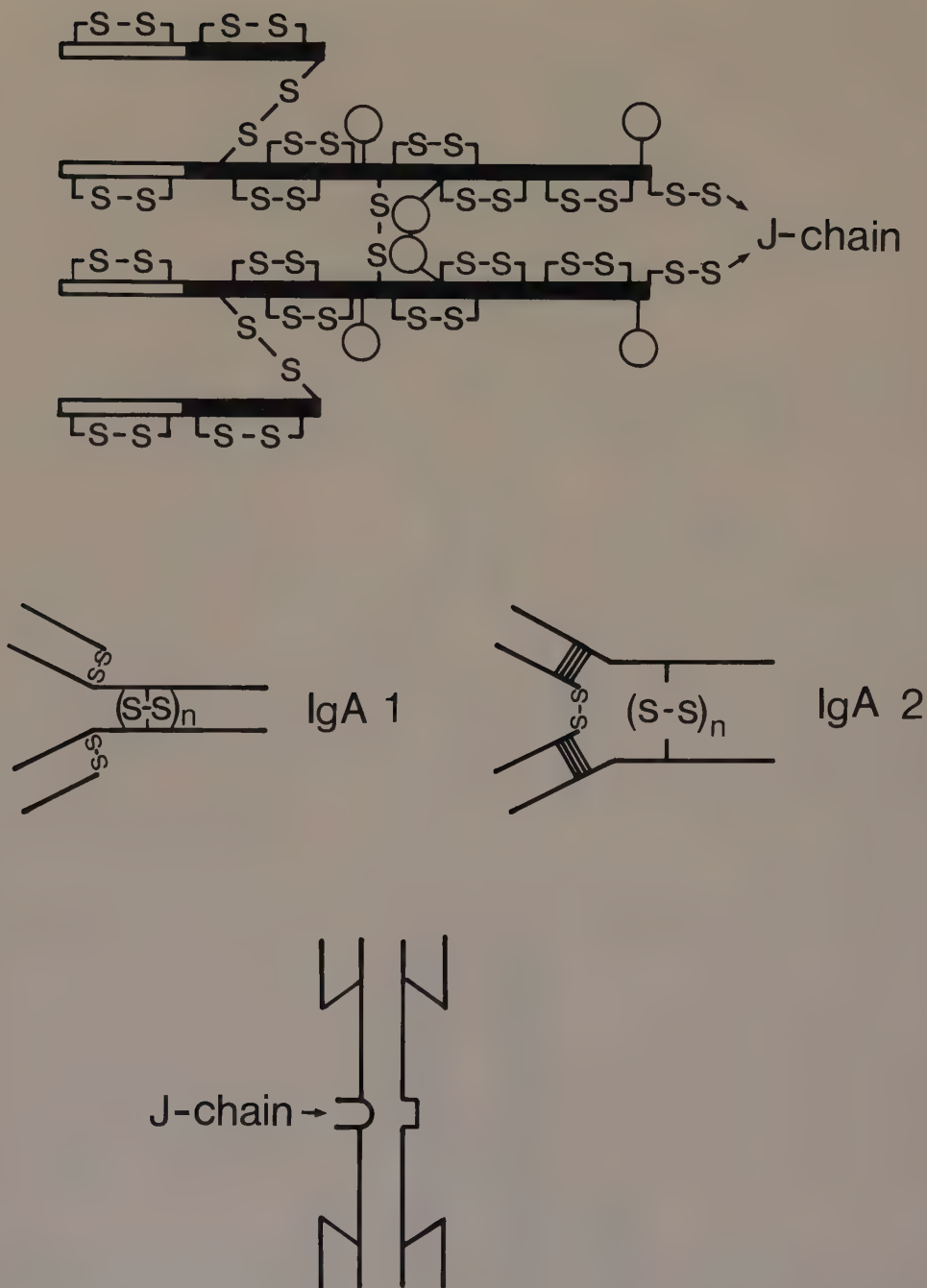


Figure 3: Top: The four chain structure of IgA.
 Centre: The arrangement of the disulphide bridges in IgA1 and 2.
 Bottom: Dimeric IgA

Antigen-antibody reactions

The antigen-antibody reaction has certain characteristics; it is specific, reversible and occurs in two different steps.

The primary reaction is the specific one between antigen and antibody. The specificity is governed by the close fit of the usually small antigenic determinants to complementary structures in the antigen binding site of the antibody. The forces holding the antigen and antibody together - hydrogen bonds, electrostatic interactions, van der Waals interactions and hydrophobic interactions - are weak, and their strength declines rapidly as the distance between the reactants increases.

The primary reaction is very fast, in fact it is one of the fastest biochemical reactions known. This has been taken as an evidence that no structural changes occur in the areas responsible for the binding itself, though this does not, of course, exclude the possibility of conformational changes occurring in other parts of the molecule. Indeed, there are results showing that such changes do occur (Chiang and Koshland, 1979; Henney and Stanworth, 1965; Henney et al., 1965; Pecht et al., 1977; Schlessinger et al., 1975).

The second reaction between antigen and antibody in solution can be manifested by a precipitation. When, for example, an increasing amount of antigen is added to a constant amount of antibody, a precipitate occurs as a result of the formation of large hydrophobic aggregates. This lattice formation is due to the divalency or multivalency of the antibody, and the multivalency of the antigen. However, the Fc parts of the antibody have also been shown to be responsible for a great deal of this precipitation (Møller, 1979; Møller and Steensgaard, 1979). Furthermore, Boyd (1973) suggested "that the precipitation is due to a lowering of the solubility by neutralization of polar groups of antibody and antigen, and concomitant steric hindrance of other polar groups of neighbouring molecules in the complex". Further addition of antigen will solubilize the precipitates due to the reversibility of the

antigen-antibody reaction, and because each antibody can be saturated with antigen. The resulting complexes are not hydrophobic enough to precipitate. The antigen-antibody reaction is thus dependent not only on the nature of the antigen and the antibody, or on the affinity of the antibody to its antigen, but also on the concentration ratios.

Rheumatoid factors

The rheumatoid factor (RF) is a characteristic immunological feature of rheumatoid arthritis (Johnson and Faulk, 1976), and is supposed to be an auto-antibody (mainly of IgM and IgG class) against autologous IgG.

RF has been shown to be rather heterogeneous in its specificity and reacts in vitro with determinants in both the $C_{2\gamma}$ and $C_{3\gamma}$ domains of IgG (Gaarder and Natvig, 1970; Natvig et al., 1972; Steward et al., 1973), and with both autologous, homologous and heterologous IgG (reviewed by Johnson and Faulk, 1976) in its native form (Allen and Kunkel, 1966; Gaarder and Natvig, 1974; Hannestad, 1968; Normansell and Stanworth, 1968), as well as in its aggregated form (Dissanayake et al., 1977; Hannestad, 1968; Henney and Stanworth, 1965). RF also reacts with antigen-antibody complexes (Aho and Simons, 1963; Ash et al., 1971; Edelman et al., 1958; Gipson and Daniels, 1975; Harboe, 1961; Lambert et al., 1978; Lamers et al., 1981; Lightfoot et al., 1969; Markenson et al., 1975; Tesar and Schmid, 1970; Van Snick et al., 1978). Furthermore, RF cross-reacts with cell nuclear components (Hannestad and Johannesson, 1976; Hannestad, 1978; Johnson, 1979; Hannestad and Stollar, 1978; Agnello et al., 1980).

Although new determinants might occur on aggregated IgG (Arai and Shimizu, 1981; Gaarder and Michaelsen, 1974), and although certain RF have a preferential binding to altered IgG (Brown and Brown, 1981, 1982), this does not necessarily mean that RF has a higher affinity to these determinants than to native IgG. The strong binding between RF and aggregated IgG is probably due to a multiple binding, rather than to a high affinity for new determinants on the IgG (Dissanayake et

al., 1977; Eisenberg, 1976; Gaarder and Natvig, 1974; Normansell, 1970). The reaction between RF and native human IgG is possibly dependent on a cross-reaction (Brown and Brown, 1981, 1982), which may be manifested by the low binding constants observed (Dissanayake et al., 1977; Eisenberg, 1976). High affinity RF are probably rapidly eliminated from the circulation (Bluestone et al., 1970; Steward et al., 1973), if indeed they ever reached it. Alternatively, they may form precipitates with the antigen at the site of the RF production (Robbins et al., 1978).

The binding of RF to IgG is often used for RF detection. So called hidden RF have to be dissociated from the bound IgG before they can be detected. Low affinity complexes between RF and IgG can be dissociated by dilution (Abruzzo and Heimer, 1974), by gel filtration (Bluestone et al., 1969), or by the addition of chemicals (Hansson and Winblad, 1978). More strongly bound RF can be dissociated from the bound IgG in acid medium (Moore et al., 1978; Falus et al., 1979). All RF IgM, whether hidden or free, seem to be detected by quantitative precipitin analysis against anti-Fc μ antiserum (Uesson and Hansson, 1981).

There are some restrictions in both the constant and the variable part of RF. These include, for example, enrichment of subclasses 1 and 4 (Gabriel and Hadler, 1981; Shakib and Stanworth, 1978), and of kappa light chains (Carson and Lawrance, 1978; Kunkel et al., 1974), and restrictions to the V $_{\kappa}$ III (Kunkel et al., 1974) and the V $_{\text{H}}$ III subgroup (Førre et al., 1977). Restrictions in the antibody combining site are exemplified by the occurrence of cross idiotypic determinants in monoclonal and polyclonal RF (Carson and Lawrance, 1978; Førre et al., 1979; Kunkel et al., 1973), and in the hypervariable region of RF (Capra and Klapper, 1976).

There are several indications of non-specific induction (Möller et al., 1980) of the RF synthesis. These include B cell activators such as the Epstein-Barr virus (Pasquali et al., 1981; Slaughter et al., 1978;

Vaughan, 1979), pokeweed mitogen (Koopman et al., 1980; Pasquali, 1981), and bacterial lipopolysaccharides (Izui et al., 1979). There are also indications of more specific induction, including a hypothesis about altered IgG stimulating B cells to produce RF (Johnson et al., 1975), and results showing that denatured IgG induce production of RF (Pisko et al., 1982).

The pathogenic role of RF is poorly understood (Johnson and Faulk, 1976; Maini, 1978; Wager, 1975), as is the role of the circulating immune complex that occurs in the serum and synovial fluid in RA.

Immune complexes and immunoglobulin complexes

Complexes formed when antibodies react with their antigens are often named immune complexes. Complexes formed when antibodies are bound to each other without the participation of antigens will be referred to here as immunoglobulin complexes. Small amounts of immune complexes occur in the serum of healthy individuals (Delespesse et al., 1980; Paganelli et al., 1979; Puskas et al., 1982). Increased amounts of immune complexes of the IgG-IgG or IgG-IgM type occur in the serum of individuals with rheumatoid arthritis (Bourke et al., 1982; Halla et al., 1979; Kunkel, 1961; Pope et al., 1975a; Roberts-Thomson et al., 1976a). RF is frequently present in these circulating complexes (Gabriel and Hadler, 1981; Jones et al., 1980; Pope et al., 1974, 1975b), and the amount of such immune complexes seems to be related to the severity of rheumatoid arthritis (Levo et al., 1981; McDougal et al., 1982). The immune complexes are assumed to initiate inflammation and tissue damage (Carter, 1973; Harris, 1982), and to be involved in the pathogenesis of RA (Harris, 1982; Ziff, 1973; Zwaifler, 1974).

The occurrence of immune complexes of IgG-IgG type (Jasin et al., 1970; Pope et al., 1974, 1975b) or immunoglobulin complexes composed of IgG (Abruzzo et al., 1970) has been attributed to the development of the hyperviscosity syndrome. This disease is characterized by high serum viscosity accompanied by various clinical symptoms such as tiredness, vertigo, gastro-intestinal bleeding and impaired vision. The

hyperviscosity syndrome is also a complication in some patients with IgA myeloma (Preston et al., 1978; Roberts-Thomson et al., 1976b). High serum content of immunoglobulin complexes composed of IgA have, for instance, been correlated to a high serum viscosity and the appearance of the hyperviscosity syndrome (Preston et al., 1978; Roberts-Thomson et al., 1976b). As the presence of these complexes does not always lead to the development of the syndrome, some property of the complexes other than the complex formation per se may be essential to the occurrence of the disease.

MATERIALS AND METHODS

Information relevant to this section will be found in the publications I - V.

RESULTS AND DISCUSSION

Dissociation of antigen-antibody complexes by RF IgM

Circulating RF occurs in a reactive form in which RF retains reactive Fab parts, and in an unreactive (hidden) form in which RF is bound to IgG in such a way that it has no Fab part that can bind to another IgG. By binding to the IgG in antigen-antibody complexes, RF IgM aggregates them and enhances their phagocytosis (Lamers et al., 1981; Van Snick et al., 1978), and RF IgM with a high affinity for IgG can quickly be eliminated from the circulation (Bluestone et al., 1970; Steward et al., 1973). Circulating RF might thus be anti-gamma globulins with low or moderate affinity for IgG. That the circulating RF has indeed a low affinity for the bound IgG is shown by the dissociation of hidden RF from the IgG on dilution (Abruzzo and Heimer, 1974), or on gel filtration at neutral pH (Bluestone et al., 1969). This may explain why two intermediate complex fractions, isolated from two RF negative sera, were found to have RF activity (III). It may also explain why RF IgM was not found in the precipitates formed in the electroimmuno assay and single immunodiffusion analyses (II), and why complexes with affinity to heat-denatured human IgG rearranged after elution from the matrix (III).

The amount of IgM reported in article II to have dissociated immune complexes is equivalent to a concentration of 7.4×10^{-2} g/l, the normal serum concentration being 0.5 - 2 g/l (Stott and Williamsson, 1982). Thus the dissociating effect of RF IgM was observed for IgM concentrations lower than those found in serum. The dissociation of antigen-antibody complexes would be obscured if high affinity RF was present. Such an effect would explain why some RF fractions had no detectable complex dissociating capacity (I). Some complexes would be aggregated by high affinity RF at the same time as others would be dissociated by low affinity RF; and, as most of the RF IgM fractions had a dissociating capacity (I), clearly the low affinity RF predominated in the RF preparations analysed.

Antigen-antibody (Ag-Ab) complexes of the type albumin/anti-albumin are solubilized at an antigen excess larger than or equal to ten times equivalence (II, Schifferli et al., 1980; Steensgaard et al., 1975; Tesar and Schmidt, 1970). Ag_2Ab and Ag_3Ab_2 complexes constitute a great part of the immune complexes in this zone (Henney et al., 1965; Steensgaard et al., 1975). RF can precipitate Ag_3Ab_2 complexes, but not Ag_2Ab complexes (Henney and Stanworth, 1965), perhaps because RF usually has a low affinity for monomeric IgG (Dissanayake et al., 1977; Eisenberg, 1976; Normansell, 1970; Normansell and Young, 1975; Steward et al., 1973). It might also be due to the arrangement of the antibodies in the complexes (Gaarder and Natvig, 1974; Lightfoot et al., 1969; Normansell, 1971); that is, the antibodies must be bound in such a way that RF IgM bind to the Fc part of more than one IgG molecule - otherwise the binding of the RF to the complexes would not be strong enough for aggregates to be formed. The dissociating effect of RF IgM was enhanced as the amounts of antigen were increased from about nine times to sixteen times equivalence (I), after which, it remained constant (I) showing that RF IgM mainly affects complexes of the Ag_2Ab type.

Complexes with the same antigen/antibody ratio may, of course, display different conformations. Proteins, cells and viruses, for example, have spectra of antigenic determinants (Wilson, 1977), and antibody populations produced against them will have different specificities and affinities (Turner, 1977). This means that RF IgM would, for example, aggregate some Ag_3Ab_2 complexes, while preventing others from growing - an effect that could become pronounced in the antigen excess zone where the antibodies were "diluted" by antigen.

The dissociating effect of RF was more pronounced when RF was added to preformed antigen-antibody complexes, than it was when RF was added before the complexes were formed (I). This agrees with results obtained by others showing that RF reacts better with antigen-antibody complexes than with free IgG (Aho and Simons, 1963; Edelman et al., 1958; Markenson et al., 1975). A possible explanation for the dissociation mechanism is that, when RF bound to the Fc part of the

antibody (Gaarder and Natvig, 1970 and 1974; Natvig et al., 1972; Steward et al., 1973) in the complexes, it sterically hinders further binding of antigen. The interaction between RF and the Fc γ part might also induce rearrangement of the complexes. Again, the binding of antigen to antibody might also be hindered if the binding of RF to the Fc part of the antibody caused a conformational change in the Fab part - in much the same way as the binding of antigen to the Fab part can induce conformational changes in the Fc part (Chiang and Koshland, 1979; Henney and Stanworth, 1965; Pecht et al., 1977; Schlessinger et al., 1975). Another possible mechanism for dissociation is that secondary bindings between complexes are affected by RF IgM, an interpretation supported by the results by Uesson and Hansson (1981), showing that RF IgM can induce a rearrangement of immune complexes formed in slight antigen excess without quantitatively affecting their antigen content. Thus RF IgM mainly dissociated secondary bindings between complexes, hindered the establishment of such bonds, or induced rearrangement of the complexes, so primary antigen-antibody bindings were probably affected to a minor extent; this is supported by the fact that RF IgM had no demonstrable effect on the binding of antigen or antibody to the corresponding immobilized antibody or antigen (II). Dissociation of primary antigen-antibody bindings would be a manifestation of low affinity between antibody and antigen; the possibility of such dissociation cannot be ruled out.

Comparison between the dissociation of antigen-antibody complexes by RF IgM (I, II), and the complement mediated effects described by Nussenzweig and his co-workers (Czop and Nussenzweig, 1976; Miller and Nussenzweig, 1975; Takahashi et al., 1977, 1978 and 1980), and by Schifferli and his co-workers (Schifferli et al., 1980 and 1982; Schifferli and Peters, 1982), reveals differences between the mechanisms. RF IgM affects soluble antigen-antibody complexes formed under such conditions that no precipitation occurs (II), while the complement effects are demonstrated on antigen-antibody complexes formed when immune precipitation occurs (Miller and Nussenzweig, 1975; Schifferli et al., 1980). Yet further differences regarding the kinetics, temperature dependence and valency of active components (I,

II: Takahashi et al., 1980; Schifferli et al., 1982), show that the dissociation of soluble antigen-antibody complexes by RF IgM and the complement effects on immune complexes discussed here are distinct features.

However, there also exist certain similarities between the effects. They all seem to be general - that is, as long as there are determinants for RF IgM or complement to react with (I; Schifferli et al., 1980 and 1982; Stassen and Beek, 1982; Takahashi et al., 1980), the effects occur irrespective of the type of antigen in the complexes, or of the origin of the antibody (I; Stassen and Beek, 1982; Takahashi et al., 1980). Furthermore, both RF IgM and complement (Takahashi et al., 1980; Schifferli et al., 1980 and 1982) dissociate secondary bonds between immune complexes, which implies that the effect of RF IgM and of complement might occur simultaneously without being recognized as separate phenomena. However, complement did not affect the results obtained here (I, II).

RF IgM could not be shown to influence the binding of antigen or antibody to the corresponding immobilized antibody or antigen, even if it could be shown to bind to the complexes (II). These results agree with those showing that RF IgM can bind to antigen-antibody complexes where the antigen has been immobilized (Markenson et al., 1975), or can bind to anti-virus antibodies bound to virus infected cells (Austin and Daniels, 1976; Gipson and Daniels, 1975) and to virus/anti-virus IgG complexes (Ashe et al., 1971; Gipson et al., 1974). Our results show that antigen-antibody complexes which are tightly bound to cell surfaces are not affected by RF in the same way as are small soluble antigen-antibody complexes, and that neither antigen nor antibody is dissociated from the complexes. This is worthy of note, since - when it is bound to these systems - RF can inhibit complement-mediated phagocytosis (McDuffie and Brumfield, 1972), inhibit cell-mediated cytolysis of virus infected cells (Austin and Daniels, 1976; Gipson and Daniels, 1975), and enhance virus neutralization with the help of complement (Ashe et al., 1971; Gipson et al., 1974).

The results reported in articles I and II show that RF may have a negative effect on the elimination of immune complexes. Immune complexes formed in gross antigen excess early in an infection might be dissociated by RF - especially if RF has low affinity for the immune complexes. Such an effect might delay their elimination (Mannik, 1980).

Interaction between normal IgM and soluble antigen-antibody complexes

In the antigen excess zone, normal IgM induced a decrease in the amount of free antigen or small soluble immune complexes when it was added to soluble antigen-antibody complexes (I). In the beginning of an infection, when gross antigen excess occurs, there are both free antigen and small soluble antigen-antibody complexes. Not only the free antigen, but also the immune complexes may be infectious (Gipson et al., 1974). Under such circumstances it is vital to aggregate and neutralize the antigen and the complexes as effectively as possible. This might be partly brought about by the multivalency of the IgM antibody, produced as the first response to antigenic challenge, and further enhanced when normal IgM interacted with small immune complexes and enhanced their aggregations.

Such an effect could, for instance, be established through hydrophobic interaction. Though IgM can bind to hydrophobic molecules such as human albumin (Harboe and Følling, 1974; Ropars et al., 1972), here the effect of normal IgM was unrelated to its affinity for human albumin, since normal IgM, both with and without this affinity, had the same effect (II). Normal IgM had the same effect on albumin-IgG complexes as on IgG-IgG complexes (I), which suggests that the aromatic or other hydrophobic groups exposed on human albumin were of less importance. This was verified when it was shown (U.-B. Hansson, unpublished observation) that normal IgM which bound to phenyl-Sepharose could be divided into two parts - one which had affinity for albumin and another which had none. It was concluded that hydrophobicity, as determined by affinity to phenyl groups, may be of general importance in the interaction with immune complexes. That hydrophobic interactions might be involved was supported by the fact

that such interactions are known to stabilize protein interactions (Chothia and Janin, 1975) and antigen-antibody interactions (Levison et al., 1970).

Mechanisms for the formation of immunoglobulin complexes

The mechanism for the formation of the intermediate IgG complexes may vary. One such complex, from a patient with polyarthritis and hyperviscosity syndrome, contained an unusual IgG which non-specifically bound by its Fc part to normal IgG (Heimer et al., 1971). The serum from another patient with hyperviscosity syndrome contained a cyclical IgG complex composed of two IgG molecules with anti-Fc γ activity bound to two other IgG molecules without anti-Fc γ activity (Gabriel and Hadler, 1981). Three sera from patients with rheumatoid arthritis contained RF IgG molecules that, by self-association, formed dimeric cyclical complexes - that is, the molecules contained both the antibody and the antigen part (Pope et al., 1974, 1975b). This type of complex was also detected by Natvig and Munthe (1975) in rheumatoid inflammatory joint tissue. The complexes in the four sera studied in paper III originated from rheumatoid and hyperviscous sera, and contained not only RF IgG but also IgG without RF activity, suggesting that self-association of rheumatoid factors was not a general mechanism for the formation of IgG complexes in these samples. Our material was also too small, however, to permit any more general conclusions to be drawn. Aggregates were formed in the rheumatoid cases and complexes were dissociated in the non-rheumatoid cases (III), when normal or myeloma IgG was added to fractions containing the intermediate complexes. This means, assuming that the added IgG functioned as antigen, that there was an antibody excess in the rheumatoid fractions and it could mean that there was an antigen excess in the non-rheumatoid fractions. Additional human albumin had no effect on complex formation, indicating that the effect produced by the additional 7S IgG was not a more general protein effect (U.-B. Hansson, unpublished note). Sixty per cent or more of the proteins in the non-rheumatoid fractions was 7S IgG (III). This IgG could be a RF with a very low affinity to the added normal IgG. Instead, the added IgG could bind to the antibodies

in the intermediate complexes. As the normal IgG was added in great excess (U.-B. Hansson, unpublished note) it was able to dissociate available complexes.

The IgA complexes analysed here (V) did not dissociate in an acid medium, but only when they were reduced with mercapto-ethanol, showing that, unlike the IgG-IgG and IgG-IgM complexes analysed in papers III and IV, these complexes were linked by disulphide bonds.

The circular dichroism spectrum of IgG complexes with affinity to heat-denatured human IgG was compared with the corresponding spectrum of monomeric IgG without RF activity isolated from the same patient, and with monomeric IgG purified from a commercial IgG preparation (IV), showing that the IgG in the complexes had conformational deviations as compared to the two monomeric IgG samples, which were identical (IV). The differences were not a result of the complex formation per se (IV), but were due to differences in the exposure of aromatic amino acids and possibly also from disulphide bonds in asymmetric environments in some of the monomers forming the complexes (IV). Interestingly, the circular dichroism spectra at 251 nm and at pH 11 of the 7S IgA and the IgA complexes showed differences similar to those of 7S IgG and IgG complexes under the same conditions (IV, V). As the molecules in both cases originated from individuals with hyperviscosity syndrome, the results could be interpreted to mean that there are structural similarities between the IgG in one patient and the IgA in another which are related to the syndrome.

The results from the hydrophobic interaction chromatography and the circular dichroism analysed indicate that the hydrophobic properties of IgA was one variable that may be significant in the development of the hyperviscosity syndrome. Though the material was too small to allow any generalizations, a comparison of the circular dichroism spectra and the hydrophobicity of IgA from four other patients with hyperviscous plasma, one of which had no syndrome, supports the theory that the hydrophobic property of the molecules in a patient with hyperviscous plasma is of importance in the development of the syndrome. U.-B.

Hansson and her co-workers found that the only IgA showing a "normal" CD spectrum at pH 11, and lowered affinity to phenyl-Sepharose and octyl-Sepharose, was the IgA originating from the patient with no symptoms of a hyperviscosity syndrome (unpublished results).

All the intermediate complex fractions analysed in paper III contained three to four times as much neuraminic acid as did normal IgG. There are results showing that sialic acids, bound to the light chains of IgG can influence the complex formation of the molecules (Hymes et al., 1979). It may be so that the carbohydrate in the Fab part is attached to the hypervariable regions (Spiegelberg et al., 1970; Sox and Hood, 1970) and thus affects the specificity of the antibody. There is a correlation between the sialic acid content of the heavy chain of hybridoma IgA from mice and antibody specificity (Matsuuchi et al., 1981). In one of their cases, the carbohydrate was attached to the hypervariable region. There was, however, no correlation between the high neuraminic acid content in the intermediate complex fractions analysed here and complex formation (III). Neither was there any correlation between the neuraminic acid content and an affinity to heat-denatured IgG (III), which might mean that in our cases the neuraminic acid was not located in the Fab part of IgG. Since the removal of sialic acids is also said to be important when glycoproteins are eliminated (Finbloom et al., 1981; Morell et al., 1971), it is also possible that the increased amount of sialic acids results in prolonged circulation time for the intermediate complexes. Further study concerning the sialic acid content in different parts of the IgG molecules in our complexes, and concerning the elimination of the complexes containing high amounts of sialic acid, will clarify the important question of the biological function of high sialic acid content.

The IgG in the intermediate complex fractions (III) demonstrated a restriction to subclass 1 in three cases, and to subclass 3 in one case. A restriction to subclass 1 of the IgG in intermediate complexes was also found by Heimer et al., (1971), and by Capra et al., (1971). Gabriel and Hadler (1981) isolated a complex containing IgG1 rheumatoid factors and IgG3 as antigen, while Shakib and Stanworth (1978)

found increased values of predominantly IgG1 RF and IgG4 RF. However, there were no indications that subclass restriction had anything to do with the formation of the complexes analysed in article III. High concentrations of antibodies to antigenic determinants located on the Fab or F(ab')₂ portions of IgG have been found in the sera of patients with rheumatoid arthritis (Heimer et al., 1982). The biological significance of these findings is just beginning to emerge. It cannot yet be ruled out that some complexes in our samples were formed to some extent as the result of a reaction between anti-epitopes and epitopes located in the F(ab')₂ part of IgG molecules. Future study should settle this.

Unique antigenic determinants

Analysis of the IgG in the intermediate complex fractions showed that these fractions contained molecules with abnormal conformations (III, IV). Concerning the IgG of patients with rheumatoid arthritis, various findings have been reported: structurally altered IgG (Watkins et al., 1972; Watkins & Swannell, 1973); deviant papain hydrolyse patterns (Watkins et al., 1970); and deviant circular dichroism spectra - when it was suggested that the anomalies were located in the hinge region (Johnson et al., 1974). In this context it should be borne in mind that it was not the purified proteins that were analysed but the pool of IgG in the sera, and thus the question whether the conformationally deviant proteins really were related to RF remained unanswered.

To identify and isolate structurally altered IgG in IgG complexes, a specific antiserum against the presumed deviant conformations would be a very useful tool. Anti-idiotypic antisera have been produced against monoclonal and polyclonal IgM RF, and against monoclonal IgG RF (Carson and Lawrance, 1978; Førre et al., 1979), but attempts to produce antisera against polyclonal rheumatoid preparations containing intermediate complexes have been less successful (Heimer et al., 1971; Johnson et al., 1975; Pope et al., 1974). If any complexes do indeed exist, containing the IgG suggested as the protein against which the

rheumatoid factors are so called auto-antibodies, it may well be the polyclonal intermediate complexes.

Paper III shows the reaction of two antisera which we succeeded in producing against two isolated intermediate complex fractions. After appropriate absorption, these antisera still precipitated proteins in the immunogenic fractions. Affinity chromatography against the $F(ab)_2\gamma$ of these antibodies clearly showed that unlike that of normal human serum protein in the three sera that could be tested from the individuals with RA and hyperviscosity syndrome, contained IgG with affinity to the antibodies (III).

As already mentioned, circular dichroism analysis showed that IgG in the intermediate complexes was conformationally anomalous (IV). Since the question now was whether the proteins with affinity to the specific antibodies might be responsible for the anomalies, proteins with the so called unique determinants were isolated from one of the sera (IV). The circular dichroism spectra of these proteins were about the same as those of the proteins in the total pool of intermediate complexes (IV; Uesson and Hansson, 1982), but there were also some differences. There was a deviation (IV) that could be due to ionization of tyrosine (Dorrington and Smith, 1972), and one deviation (IV) that might be related to β -structures of the molecules (Dorrington and Smith, 1972), the latter having also been observed in an antibody preparation (Bear et al., 1974) when it was taken to be due to differences in hydrogen bond position and orientation, rather than to differences in amount of β -sheet."

Attempts were made to localize the unique determinant(s) within the IgG molecules, and in one case the determinants were found in the $F(ab')_2\gamma$ part (III). Later experiments (Uesson and Hansson, 1982) have localized the determinant(s) to the Fab part of the unique IgG in another patient. The unique molecules constituted a minor part of the molecules within the intermediate complexes (Uesson and Hansson, 1982) with RF activity (III). This could mean that the molecules with the unique determinants were not RF, but IgG bound to RF.

The unique determinants were detected in the $F(ab')_2$ part of IgG. Cross-reaction with the intermediate complex IgG fractions from different individuals showed that the same determinants were found in the IgG from different people. The question of their biological function remains unanswered. The IgG with the unique determinants could be anti-idiotypic antibodies against idiotypic determinants in RF; they could be anti-idiotypic antibodies to IgG with some unknown antibody specificity or, again they could be antibodies to some unknown antigen. Though the possibilities are many, and the mechanisms might be complex, future study will doubtless answer these questions. One thing, however, seems clear, namely that their conformation is abnormal. The proposal that the unique IgG molecules occur in the serum from individuals with rheumatoid arthritis received some support from the results of a blind investigation of eleven sera from individuals with various diseases of the joints, of which six had RF; it was shown that the occurrence of the immunoglobulins with the unique determinants reported here (III, IV) was associated with rheumatoid arthritis (U.-B. Hansson, unpublished observation).

CONCLUDING REMARKS

RF IgM was shown to dissociate soluble antigen-antibody complexes formed in gross antigen excess. This novel effect attributed to RF was due to the presence of low affinity RF.

Normal IgM was shown to have a previously unknown capacity for enhancing the precipitation of soluble immune complexes formed in gross antigen excess. The effect, which in vivo might increase the effectiveness of the aggregation of small immune complexes, was probably due to a hydrophobic interaction between normal IgM and the immune complexes.

IgG in sera from patients with rheumatoid arthritis or hyperviscosity syndrome (or both) was shown to contain unique antigenic determinants, which could be located to the $F(ab')_2\gamma$ part and which were not related to RF. These new findings show that the conformation of IgG in the immune complexes deviates from that of normal IgG.

Both IgA from hyperviscous serum; and IgG with the unique determinants were shown to produce abnormal circular dichroism spectra, demonstrating deviations in the exposure of aromatic amino acids and possibly in the microenvironment of disulphide bridges. The IgA was, moreover, found to be more hydrophobic than normal IgA. These findings provide new prospects with regard to the relation between hyperviscosity in serum and the hyperviscosity syndrome.

The findings are not merely of interest in elucidating the mechanisms responsible for the formation of circulating immune complexes and their role in the pathogenesis of rheumatoid arthritis; they are also of importance in understanding the mechanisms and structures responsible for the development of the hyperviscosity syndrome, and will even be useful in elucidating the biological effects of RF and the origin of the antigen for RF.

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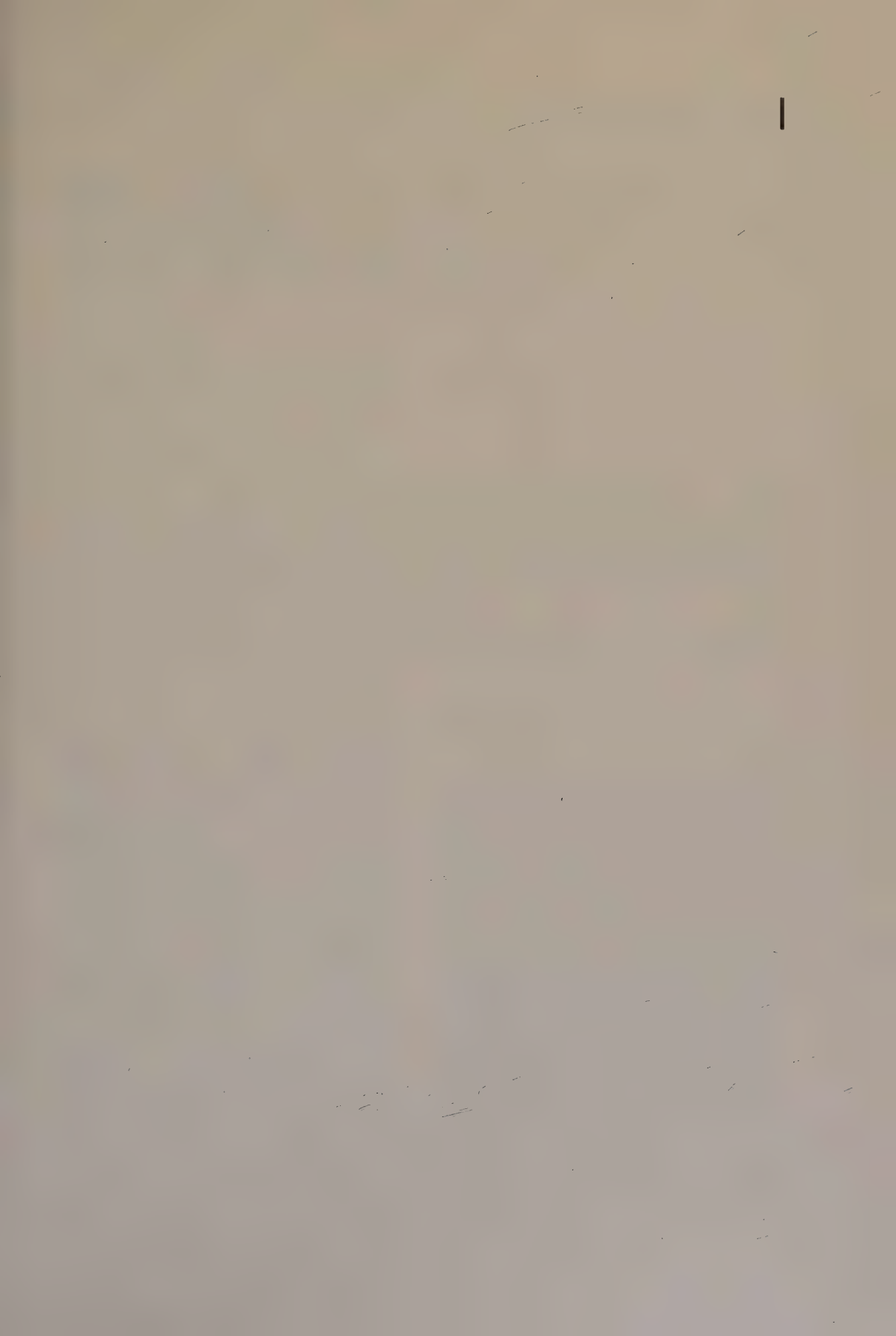
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Effect of IgM Fractions with or without Rheumatoid Factor Activity on Antigen–Antibody Reactions

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Alkner, U. & Hansson, U.-B. Effect of IgM Fractions with or without Rheumatoid Factor Activity on Antigen–Antibody Reactions. *Scand. J. Immunol.* 10, 119–125, 1979.

The effect of isolated IgM fractions, with or without rheumatoid factor (RF) activity, on reactions between human albumin and rabbit anti-human albumin, human IgG and rabbit anti-human IgG, and tetanus toxoid and human anti-tetanus toxoid was assessed in the antigen excess zone. RF-active fractions increased and RF-negative IgM fractions decreased the amount of free antigen. The immune complexes that were affected by RF IgM fractions differed in composition from those affected by normal IgM fractions.

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Circulating immune complexes act pathogenically by mediating the release of inflammatory agents [4]. The pathogenicity of the complexes may depend on their composition; both solubilization and aggregation might therefore affect their pathogenicity.

IgM rheumatoid factor (RF) aggregates immune complexes *in vitro* [1, 6, 16], which is in contrast to the fact that IgM RF and immune complexes are often found to coexist *in vivo* [20]. Monovalent antibody fragments directed against antibody in immune precipitates solubilize immune complexes [17]. IgM RF is thought to be an antibody directed against IgG, but no dissociating effect of RF has been reported.

The purpose of the present investigation was to ascertain how isolated IgM fractions with or without RF activity affect antigen–antibody reactions, in the hope that it would elucidate the biological function of IgM RF and normal IgM and the way these proteins may affect immunological protein quantitation in human serum.

MATERIALS AND METHODS

Sera. Fourteen macroglobulinaemic sera (M1–M14), twenty-one sera (RA1–RA21) from patients with symptoms of rheumatoid arthritis (RA), and three sera (N1–N3) from registered blood donors were used. Sera M14, RA12–RA21, and N2–N3 were fresh, whereas the remaining sera had been stored at -20°C for at least 3 months. Sera M13, RA7–RA11, RA15 and RA20 showed RF activity.

Antisera. Rabbit antiserum against whole normal serum was from Hyland Laboratories Inc., Los Angeles, Calif., USA, and the human anti-*Clostridium tetani* toxoid (16.5% IgG solution) from the National Bacteriological Laboratory, Stockholm, Sweden. Double immunodiffusion against appropriate antisera showed that the anti-tetanus preparation contained only IgG. Rabbit antisera against α -lipoprotein and β -pre- β -lipoprotein were a gift from Dr Bengt Johansson, Department of Clinical Chemistry, University of Lund, Lund, Sweden. Rabbit antisera against α_2 -macroglobulin and the complement factor C3 were from the Department of Clinical Chemistry, The General Hospital of Malmö, Malmö, Sweden.

All other antisera were prepared in our laboratory by hyper-immunizing rabbits with the appropriate component in complete Freund's adjuvant. Before use, the antisera were made monospecific by adsorption.

The IgG in the antisera against human IgA, IgM,

albumin and Clq was precipitated by 1.84 M ammonium sulphate and dissolved in 0.9% NaCl. Double immunodiffusion against appropriate antisera revealed no Clq or C3 in these preparations.

Electrophoretic and immunological methods. Agarose gel electrophoresis, single immunodiffusion [14], double immunodiffusion [18], immunoelectrophoresis [22], and electroimmunoassay [12] were run on 0.8% agarose (Miles-Seravac, Maidenhead, Berks., England) in 0.075 M barbital buffer, 2×10^{-3} M calcium lactate, pH 8.6 (20 V/cm for the electrophoretic methods).

Rheumatoid factor activity. RF activity was in all cases determined by a sheep erythrocyte agglutination method that also detects hidden RF [8]. The test was considered positive when the titre was 32 or more. Latex fixation test [8] was used on thirteen of the fourteen macroglobulinaemic samples. The test was considered positive when the titre was 40 or more.

Isolated serum proteins. The human albumin (for intravenous use) and IgG (for human use) were from AB Kabi, Stockholm, Sweden. Monomeric IgG was prepared from the IgG solution by gel filtration on Sephadex G-200.

Human β -lipoproteins were from the Department of Clinical Chemistry, University of Lund. The *Clostridium tetani* toxoid was a gift from the National Bacteriological Laboratory. The toxoid components migrated like α_1 -components on agarose gel electrophoresis.

Clq was prepared from one human serum containing three times the normal amount of Clq by filtration on heat-denatured human IgG (63°C, 30 min) coupled [5] to Sepharose 6B. Clq was eluted with 0.075 M Tris, 0.2 M 1.4 diaminobutane, 10^{-3} M CaCl_2 , pH 11.0. The precipitate formed after dialysis against 0.04 M NaCl, 10^{-3} M CaCl_2 , for 6 h, was dissolved in 0.8 M NaCl, 10^{-3} M CaCl_2 and dialysed further against 0.075 M Tris buffer, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1. A heat-inactivated (63°C, 30 min) normal human serum regained its ability to lyse sheep erythrocytes coated with antibodies when the Clq preparation was added.

IgM was isolated by gel filtration (0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0) either on Sephadex G-200 or on Biogel-A 1.5 M (M2, 9, 11). The M6 and M10 fractions were also purified by preparative electrophoresis in 1% agarose. All fractions were concentrated by vacuum dialysis in collodium bags (Sartorius Membrane filter, GmbH, Göttingen, West Germany) against 0.05 M sodium phosphate, 0.5 M NaCl, 0.1 M glycine buffer, pH 7.0, to the volume of the serum applied.

Affinity chromatography was performed on Sepharose 6B coupled [5] with immobilized heat-denatured (63°C, 30 min) human IgG. The protein eluates were assayed with light absorbency at 280 nm ($E_{1\%}^{1\text{cm}}$ (280 nm)=12.5). The proteins were first eluted with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1 (Tris buffer), then with 1 M NaCl, and finally with 0.075 M Tris, 0.2 M 2-aminobutane, 10^{-3} M CaCl_2 , pH 11.0 (2-aminobutane buffer). The proteins were concentrated by vacuum dialysis with collodium bags against the Tris buffer made 0.1 M in respect to glycine, to the original volume of the serum applied.

Quantitation of proteins in the isolated IgM fractions. IgM was determined by single immunodiffusion against

anti-IgM on reduced samples (0.1 M β -mercaptoethanol, 4°C overnight). Normal human serum, assumed to contain 100 mg IgM/100 ml [10, 11], was used as a standard. The amount of other proteins was calculated as the difference between the total protein concentration ($E_{1\%}^{1\text{cm}}$ (280 nm)=12.5) and the concentration of IgM. These proteins were identified by double immunodiffusion and immunoelectrophoresis.

Effect of IgM fractions on antigen-antibody reactions. Human albumin/rabbit anti-human albumin (albumin system), human IgG/rabbit anti-human IgG (IgG system) and tetanus toxoid/human anti-tetanus toxoid (tetanus system) were used. The analyses were performed in the antigen excess zone by electroimmunoassay.

A regression line for pure albumin was constructed by analysing different dilutions of albumin with electroimmunoassay. The agarose on the plates contained 25 μ l anti-albumin per 25 ml gel.

The effect of different mixing procedures of antigen (Ag), antibody (Ab), and IgM fraction on the results obtained on electroimmunoassay was determined for the following combinations: (1) $\text{Ag} + \text{Ab} \xrightarrow{\text{IgM}}$; (2) $\text{Ab} + \text{IgM} \xrightarrow{\text{Ag}}$; (3) $\text{Ag} + \text{IgM} \xrightarrow{\text{Ab}}$; and (4) $\text{Ag} \xrightarrow{\text{IgM}}$; that is, in procedure 1 the IgM fraction was added after Ag and Ab had been mixed. Mixtures of type 4 contained no antibodies.

Procedures 1 and 3 were tried for the albumin and IgG systems, and all four for the tetanus system.

Mixing and evaluation procedures. A 30 μ l to 70 μ l (five dilutions) sample of 0.01% albumin or a 40 μ l to 60 μ l sample (three dilutions) of 0.02% IgG was mixed with a 30 μ l IgM ($\sim 10 \mu\text{g}$ IgM) fraction (30 μ l 0.9% NaCl in the standard series) and kept at 4°C for 1 h. Then, 80 μ l anti-albumin (diluted 75 times) or 50 μ l anti-IgG (diluted 5 times) was added, and the volume made up to 300 μ l with 0.9% NaCl.

A 30 μ l to 100 μ l (three dilutions) sample of tetanus toxoid (diluted 50 times) and 30 μ l of the antibodies (diluted 100 times) were mixed and kept at room temperature for 1 h. A 30 μ l IgM ($\sim 10 \mu\text{g}$ IgM) fraction (30 μ l 0.9% NaCl in the standard series) was added, and the volume made up to 300 μ l with 0.9% NaCl.

The mixtures in all three systems were analysed after 10 min at room temperature.

The standard and sample series for each IgM fraction were run on the same agarose plate. The agarose on the plates contained 25 μ l anti-albumin or anti-IgG or 100 μ l anti-tetanus toxoid per 25 ml gel. The albumin and tetanus electroimmunoassay were run at 20 V/cm for 4 h; the IgG system for 10 h. All the plates were stained with Amido Black. For each plate the equation for the regression line of the standard series was calculated by regression analysis, and the regression coefficient compared to the corresponding coefficient of the regression line for pure albumin. The amount of 'free' antigen ($\mu\text{g/ml}$) in the sample series was found by interpolation to the standard regression line on the same plate as the sample. The differences, d ($\mu\text{g/ml}$), were calculated by subtracting the concentration of the standard from that of the corresponding sample containing IgM. The mean of all the differences for each sample ($\bar{d}$) was compared with a 95% confidence interval to determine

whether the IgM fraction significantly affected the antigen-antibody system:

$$|\bar{d}| \geq \frac{t_2 \cdot s}{b} \sqrt{\left(\frac{1}{n}\right)}$$

t_2 = Student's distribution; s = standard deviation (mm); b = regression coefficient (mm $\times$ ml/ μ g); n = number of applications (10 in the albumin system, 6 in the IgG system, and 15 in the tetanus system).

A significant change in the amount of free antigen and/or soluble antigen-antibody complexes was defined as $|\bar{d}| > 95\%$ confidence interval. Enhancement/reduction of the amount of free antigen was referred to as increase/decrease.

Lipase treatment. An RF-positive IgM fraction containing lipoproteins, isolated from a pool of seven RF-active sera, was treated with 0.1% phospholipase C, type I (Sigma, St Louis, Mo., USA) for 1 h [3]. The reaction was inhibited by addition of 0.1% lecithin.

RESULTS

On electroimmunoassay, the regression line for pure albumin was $l = 1.2 \times C$, where l denoted the height of the precipitin peak (mm) and C the concentration of added albumin (μ g/ml). The same regression coefficient was obtained for the mixture of albumin and anti-albumin ($l = 1.2 \times C - 7.5$) used as standard in the albumin system. The standard deviation was 0.52 μ g/ml in the albumin system, 0.84 μ g/ml in the IgG system, and 1.2 (mm) in the tetanus system (the concentration of tetanus toxoid was unknown). The relative amount of antigen added that had to be released/bound to produce an increase/decrease was 2% (23 μ g/ml antigen added) to 5% (10 μ g/ml) in the albumin system, 2% (40 μ g/ml) to 3% (27 μ g/ml) in the IgG system, and 11% (peak height of 11 mm) to 60% (2 mm) in the tetanus system.

Reaction times longer than 10 min at room temperature or incubation at 37°C overnight in the albumin system did not alter the results obtained.

IgM might bind albumin (Ag in the albumin system) and IgM RF might bind human IgG (Ag in the IgG system and Ab in the tetanus system) or rabbit IgG (Ab in the albumin and IgG systems). The influence of different mixing sequences of IgM, Ag and Ab was therefore determined in all three systems for one IgM RF and one normal IgM fraction. IgM RF gave a more pronounced increase and normal IgM a more pronounced decrease when Ab was added to a mixture of Ag and IgM

(Ag + IgM $\xrightarrow{\text{Ab}}$) than when IgM was added to a mixture of Ag and Ab (Ag + Ab $\xrightarrow{\text{IgM}}$).

In the rest of this work the mixing procedure Ag + IgM $\xrightarrow{\text{Ab}}$ was used for the albumin and IgG systems and the procedure Ab + Ag $\xrightarrow{\text{IgM}}$ for the tetanus system.

The macroglobulinaemic samples showed a negative latex fixation test and lacked affinity for heat-denatured human IgG coupled to Sepharose 6B.

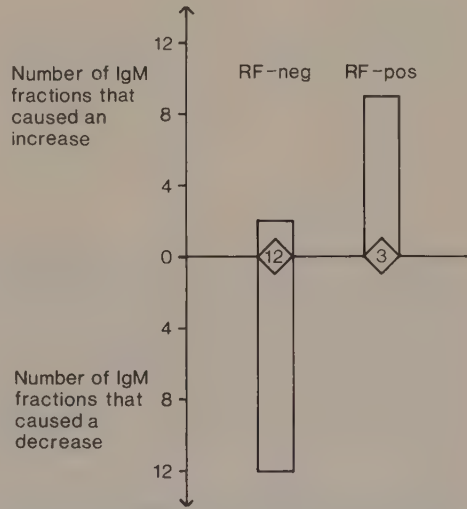


FIG. 1. Correlation between rheumatoid factor (RF) activity of the IgM fraction (isolated from fourteen macroglobulinaemic, twenty-one rheumatoid arthritis and three normal sera) and effect of the IgM fractions on the albumin system. The figures in the squares on the x-axis denote the number of IgM fractions that had no demonstrable effect on the albumin system. A significant change in the amount of antigen (increase, decrease) was defined $|\bar{d}| > 95\%$ confidence intervals (see Materials and Methods). The two RF-negative fractions that gave an increase originated from RF-positive sera.

The effect of the isolated IgM fractions on the albumin system is illustrated in Fig. 1. 75% of the RF-positive IgM fractions caused an increase in the albumin system, whereas 46% of the RF-negative IgM fractions (70% of these originating from RF-negative macroglobulinaemic and normal sera) had the opposite effect. The two RF-negative fractions that caused an increase originated from seropositive RA sera,

which means that only RF-positive IgM fractions or IgM fractions originating from sero-positive sera caused an increase in the albumin system.

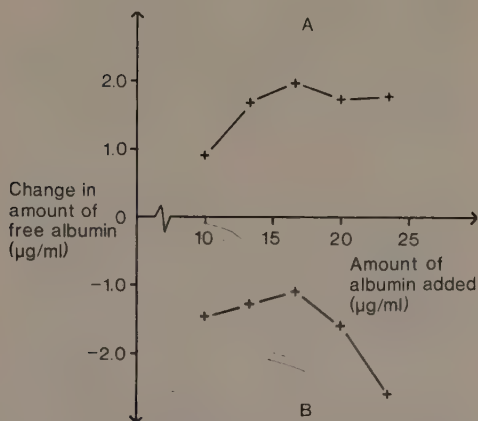


FIG. 2. The differences in amount of free albumin between standard and sample series of the corresponding dilutions of the albumin system are summarized and the mean values plotted against the amount of albumin added. Differences of 0.1 µg/ml were significant. (A) The values for eleven IgM fractions that caused an increase. (B) The values for the twelve IgM fractions that caused a decrease.

In Fig. 2, the change in the mean amount of albumin (µg/ml) is plotted against the amount of added albumin (µg/ml) for the eleven IgM fractions that elicited an increase and for the twelve that caused a decrease in the albumin system. The increase in the amount of albumin was proportional to the amount added when the concentration of albumin was less than 17 µg/ml. In the remainder of the antigen excess zone the amount of free albumin was not significantly changed. For the IgM that caused a decrease, the effect was the reverse. The amount of free albumin was constant up to 17 µg/ml of added albumin, after which it decreased.

As expected (RF fractions also contained normal IgM), no correlation was found between the RF titre of the IgM fractions and the size of the increase in peak heights noted in the albumin system.

To find out whether any correlation existed between the results in the albumin system with

those obtained in an IgG system, six RF-negative (four macroglobulinaemic and two fractions from RA sera) and one RF-positive IgM fraction, all lacking IgG, were analysed with the IgG system. All fractions had identical effects in the IgG and albumin systems. The only fraction that caused an increase was the RF-positive one. The increase was observed in the cathodal precipitin peak. The RF-negative fractions caused a decrease in the height of both the anodal and the cathodal precipitin peak.

Three RF-positive IgM fractions that had produced an increase in the albumin system were also analysed in the tetanus system. All three produced an increase in the amount of 'free' tetanus toxoid.

The mean content of IgM was 0.29 mg/ml in the macroglobulinaemic IgM fractions, 0.37 mg/ml in the RA IgM fractions, and 0.36 mg/ml in the normal IgM fractions. The mean contents of proteins other than IgM were 28%, 72% and 38% for the macroglobulinaemic, RA, and normal IgM fractions, respectively.

The IgM fractions from the RA sera and the normal sera migrated at the same rate on gel electrophoresis, and all had a diffuse γ zone. The macroglobulinaemic preparations displayed distinct bands in the γ_1 - γ_3 regions.

Most of the IgM fractions contained α_2 -macroglobulin and some contained IgA, IgG, α - and β -lipoproteins, Clq, or C3. Some representative results with regard to the presence of some of the proteins in the IgM fractions and the effect of the respective IgM fractions are given in Table I. There were no indications that the 'impurities' affected the results obtained in this investigation.

Addition of isolated Clq to the albumin system itself or to the albumin system after addition of IgM RF (two IgM RF fractions that elicited no effect) did not alter the results obtained.

Four RF-positive IgM fractions that induced an increase and contained Clq were analysed in the albumin system after inactivation (63°C, 30 min) of Clq. Two of the fractions had the same effect after this treatment as before, whereas two had no effect.

The frequency of lipoproteins was higher in samples that gave an increase than in those that gave a decrease (Table I, C). Lipase treatment of an RF-positive IgM fraction did not alter the increase noted in the albumin system. Addition

TABLE I. Effect of additional IgM fraction on the albumin system in relation to its content of non-IgM proteins

(A)			
No. of samples	Effect	α_2 -Macro-globulin	Serum proteins other than IgM and α_2 -M
1	Increase	+	0
2	No change	+	0
6	Decrease	+	0
(B)			
		Clq	C3
3	Increase	0	0
5	No change	0	0
9	Decrease	0	0
3	Increase	+	0
4	No change	+	0
1	No change	0	+
5	Increase	+	+
5	No change	+	+
3	Decrease	+	+
(C)			
		α - and β -lipoproteins	
10	Increase	+	
7	No change	+	
4	Decrease	+	

of isolated β -lipoproteins to the albumin system itself did not alter the results, even when β -lipoproteins were added in a concentration 3 times as high as that in normal human serum.

DISCUSSION

The sensitivity of the electroimmunoassay was within the limits reported by others [13]. The regression lines for pure albumin and for a mixture of albumin and anti-albumin had identical regression coefficients, which showed that the amount of complex-bound antigen was constant in all standard dilutions (no IgM added) and that the Ag-Ab complexes did not influence the results obtained with the electroimmunoassay, although the net charge of the antigen affects the peak height [7].

Some of the RF-positive sera gave RF-negative IgM fractions, which indicated that our RA sera, like those studied by others

[19, 26], contained IgG RF or that RF-active components were denatured on isolation. Freezing also denatured RF, indicated by the observation that two out of eleven frozen (RF-positive before freezing), but eight out of ten fresh (RF-positive) RA sera gave RF-positive IgM fractions. The RF activity decreased when the purity of the IgM increased.

To minimize the denaturation of the RF-active components, the IgM fractions were not highly purified and thus contained other serum proteins. We found no correlation between the existence of non-IgM proteins and the effects observed in the antigen-antibody systems. The C3-dependent solubilization of immune precipitates [17, 23], which is enhanced by the classical pathway for complement activation [24], was not involved. Some of our fractions that contained Clq and C3 gave a decrease, whereas others that contained neither gave an increase. The albumin and tetanus systems did not themselves contain Clq or C3.

The release of antigen in the Ag-Ab system was correlated to the presence of RF activity in the IgM fractions. IgM RF has been thought to be an antibody that reacts with homologous and heterologous IgG. The IgM RF fractions studied here released antigen and had an effect similar to that of monovalent antibody fragments (Fab) that were directed against the Ab part of Ag-Ab complexes and solubilized immune precipitates [17]. The three Ag-Ab systems were studied from the low to the high antigen excess zone and showed an increase when IgM RF fractions were added. This effect was independent of whether rabbit or human IgG was used as antibody or antigen. In the albumin and IgG systems the Ab was of rabbit origin and the Ag of human origin whereas the Ab in the tetanus system was of human origin. Our results thus indicated that IgM RF functioned as an antibody to IgG in the antigen-antibody complexes.

A straight line (change in amount of free albumin=constant) was not obtained in the albumin system containing IgM RF when the amount of albumin released was plotted against amount of albumin added, which would have been the case if IgM RF were bound to free antibody (the amount of antibody was constant). The amount of released albumin increased when the amount of added albumin was 10-17 $\mu\text{g/ml}$ but was constant from 17 to 23 $\mu\text{g/ml}$. This indicated that IgM RF reacted with immune complexes of a special composition, the concentration of which increased up to 17 $\mu\text{g/ml}$ of albumin added and then remained constant.

Thus IgM RF mainly reacted with the Ag-Ab complexes. This is in accordance with the results obtained by others, showing that RF reacts better with Ag-Ab complexes than with native IgG [2, 6, 16] and that the binding of RF to Ag-Ab complexes is most obvious in the antigen excess zone [6, 25]. The decrease in peak heights obtained when macroglobulinaemic or normal IgM was added to the Ag-Ab systems was striking because it did not reflect a reaction between Clq and immune complexes, since Clq was found only in 27% of the fractions producing a decrease. The decrease was smallest in that part of the antigen excess zone where Clq might have affinity for immune complexes [25]. The decrease did not reflect any binding between IgM and albumin, even if such a binding does sometimes occur [9, 15, 21], since no straight line

(change in amount of free albumin=constant) was obtained in mixtures containing IgM when the decrease in amount of free albumin was plotted against the total amount of albumin added.

It was also of interest that the immune complexes affected by normal IgM differed in composition from those affected by IgM RF. Normal IgM reacted with complexes whose concentration was constant in the first part of the antigen excess zone but seemed to increase with amount of antigen added in the gross antigen excess zone.

IgM RF has been shown to raise the amount of precipitates in Ag-Ab systems [1,6,16] but released antigen from Ag-Ab complexes in this study. If IgM RF had this effect *in vivo*, it might prolong the period of antigen in excess and increase the amount of circulating intermediate complexes, especially when poor-affinity antibodies are produced in response to the antigenic challenge. The fact that circulating intermediate complexes are common in RA [20] might perhaps be explained by this biological property of the RF.

Normal IgM had an opposite effect: in the antigen excess zone the reactions between Ab (IgG class) and Ag resulted in a decrease in the amount of free Ag. Nonspecific normal IgM might thus act as a helper in the elimination of antigenic molecules by facilitating the binding between antigen and its corresponding IgG antibody.

In conclusion, it would appear that the presence of IgM RF may favour the formation of immune complexes, whereas the presence of 'normal IgM' may favour the elimination of complexes.

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DISSOCIATION OF SOLUBLE ANTIGEN-ANTIBODY COMPLEXES

BY RHEUMATOID FACTOR IgM

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Short title:

Dissociation of Ag-Ab by RF IgM

SUMMARY

Intact RF active IgM dissociated soluble antigen-antibody complexes formed in the antigen excess zone, while trypsin-digested protein did not. The dissociation mechanism involved an interaction between the RF IgM and the Fc γ of antibodies in the complexes. RF active IgM had no demonstrable effect on antigen-antibody complexes when the antigen or the antibody had been immobilized. This was true irrespective of whether the experiments were performed in the antigen or in the antibody excess zone, and despite binding between RF IgM and the immobilized proteins.

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INTRODUCTION

It has been reported that RF active IgM affects antigen-antibody complexes formed in the antigen excess zone, causing dissociation, while normal IgM has the reverse effect (2). Both a dissociating effect of immuno precipitates (4) and inhibition of the precipitation of soluble antigen-antibody complexes formed in antibody excess or a moderate antigen excess (15) has been demonstrated for complement. It has been shown that RF IgM aggregated immune complexes formed in the presence of a moderate antigen excess (16), whereas no such effect occurs when the soluble complexes are formed in the presence of a gross antigen excess (16). We need to know more about the effect of RF IgM and normal IgM on both soluble and insoluble immune complexes; biologically and functionally may well be as important as the effect of complement. Here we have ascertained the effect of IgM on soluble and immobilized antigen-antibody complexes. It was found that RF IgM dissociated soluble complexes formed in the antigen excess zone and that RF IgM had no demonstrable effect on immobilized complexes despite binding to the complexes.

MATERIALS AND METHODS

Sera Serum from one patient with seropositive rheumatoid arthritis (RF), and normal human serum (NS; serum pool from 50 blood donors) was used. The sera had been stored at -80°C (RF) or at -20°C (NS).

Antisera Anti-IgG, anti-IgM, and anti-albumin were prepared at our laboratory by hyper-immunizing rabbits with the corresponding human antigens in complete Freund's adjuvant. Before use, the antisera were made monospecific by appropriate adsorption. Anti-IgA was obtained from Miles Laboratories Ltd, England; anti-C1q, anti-C3c and anti-C3d from Nordic Immunological Laboratories B.V., The Netherlands; anti-C3b, anti- α_2 -macroglobulin, anti-sheep erythrocytes from the Department of Clinical Chemistry, Malmö General Hospital, Sweden and anti- α -lipoproteins, anti- β -lipoproteins, and anti- κ light chains from the Department of Clinical Chemistry, University of Lund, Sweden. All the above mentioned antisera were of rabbit origin. Goat antiserum against human C3a was from Cappel Laboratories, USA, swine antiserum against rabbit IgG(Fc) was from Nordic Immunological Laboratories and sheep antiserum against rabbit IgG(Fab) was from Seward Laboratory, England.

Isolation of IgG from antisera Specific anti-albumin IgG antibodies were isolated from antiserum by affinity chromatography to immobilized human serum albumin, followed by

repeated gel filtration on Sephacryl S-300 (Pharmacia, Sweden) equilibrated with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. IgG from the anti-IgM antiserum was obtained by precipitation with 1.84 M ammoniumsulphate followed by gelfiltration on Sephacryl S-300. The $F(ab')_2\gamma$ fragments obtained by pepsin digestion of this IgG and purified by gelfiltration on Sephacryl S-200 (Pharmacia, Sweden), were used to isolate IgM by affinity chromatography. IgG from the anti-sheep erythrocyte antiserum was purified by DEAE-Sephacel (Pharmacia, Sweden) equilibrated with 0.02 M sodium phosphate, pH 8.0.

Isolation of albumin Human albumin (Calbiochem-Behring Corp., Switzerland; Albumin (human), A-grade) purified by gel filtration on Sephacryl S-200 was immobilized. Specific anti-albumin IgG was isolated from antiserum by affinity chromatography. Albumin from normal human serum was in turn isolated by affinity chromatography to these immobilized specific IgG antibodies. The albumin so obtained was further purified by gel filtration on Sephacryl S-200.

Isolation of IgM IgM, isolated from serum by affinity chromatography on immobilized anti-IgM $F(ab')_2\gamma$ fragments, was further purified by repeated gel filtration on Sephacryl S-300 in alkaline medium (0.075 M Tris, 0.2 M 2-aminobutan, 10^{-3} M $CaCl_2$, pH 11.0) to dissociate immunoglobulin complexes (6). Both IgM with and IgM without affinity to albumin were isolated from the

purified IgM by affinity chromatogry to immobilized albumin. The purity of the IgM fractions, and their content of proteins other than IgM, was determined by double immunodiffusion and immunoelectrophoresis against the appropriate antisera.

Affinity chromatography Both albumin (3 mg/ml gel), anti-albumin (10 mg/ml gel), and anti-IgM F(ab')₂γ fragments were immobilized (3) to Sepharose CL-4B. Elution was done in successive steps with 1. 0.075 M Tris, 0.075 M NaCl, 10⁻³M CaCl₂, pH 8.1; 2. 1M NaCl; and 3. 0.075 M Tris, 0.2 M 2-aminobutane, 10⁻³M CaCl₂, pH 11.0. Proteins with affinity to the matrix were eluted with buffer 3.

Protein quantitation was performed with the Folin phenol method (10).

Immunological methods and electrophoresis Single radial immunodiffusion (11), double immunodiffusion (12), immunoelectrophoresis (14), electroimmuno assay (9), and gel electrophoresis were run in 0.8% agarose gel in 0.075 M barbital buffer, 2 x 10⁻³M calcium lactate, pH 8.6. The determination of C1q by double immunodiffusion was run in 0.8% agarose gel in 0.05 M cacodylate, 0.09% NaCl, 0.01 M EDTA, pH 7.2 (1). The plates were stained with Coomassie Brilliant Blue.

Enzymatic digestion Trypsin (Sigma, USA) digestion of IgM was performed as described by Plaut and Tomasi (13), and pepsin (Sigma, USA) digestion of IgG as described by Turner et al. (18).

Rheumatoid factor (RF) activity was determined using the Latex fixation test as described by Hansson and Winblad (7).

Radioactive labelling was done by the lactoperoxidase method (17). The labelled protein was purified on a PD 10 column (Pharmacia, Sweden) equilibrated with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0.

Quantitative precipitin curve To 2.6×10^{-3} μ mole of pure anti-human albumin IgG was added 3.4×10^{-5} - 3.1×10^{-3} μ mole pure human albumin. The mixtures were incubated at room temperature in 0.9% NaCl for 9 h, the precipitates were washed with 0.9% NaCl and dissolved in 2 M NaOH, and the amount of precipitated protein was determined by measuring the light absorbancy at 280 nm.

Effect of IgM on antigen-antibody reactions

Soluble system To five dilutions of pure human albumin (6.4×10^{-5} - 14.8×10^{-5} μ mole) was added 2.3×10^{-5} μ mole of IgM, and the mixtures were incubated for 1 h at 4°C. 5.5×10^{-5} μ mole of anti-albumin IgG was added and the mixtures were incubated in 0.9% NaCl (a total volume of 300 μ l) for 15 min at room temperature. Then the mixtures were analysed by electroimmuno assay (2), or by single radial immunodiffusion. Standard series, in which the IgM

was replaced by 0.9% NaCl, were always run in parallel with the samples, and on the same plate. The height of precipitin peaks in the electroimmuno assay and the area of the precipitin rings in the single radial immunodiffusion were measured, and the significance of the differences between sample and standard series were determined using t-statistics with a 95% confidence interval (2).

Immobilized systems 1. Antibody in excess: 50 μ l

Sepharose CL-4B containing 7.6×10^{-4} μ mole immobilized human albumin in 0.05 M sodium phosphate. 0.5 M NaCl, 0.1% gelatin, pH 7.0, was mixed with 1.4×10^{-4} μ mole of 125 I labelled anti-albumin. 150 μ l of the immobilized albumin (2.3×10^{-3} μ mole) was found to have bound 80% (1.1×10^{-4} μ mole) of the added anti-albumin; 2 and 3.

Antigen in excess: 25 μ l Sepharose CL-4B containing 5.2×10^{-4} μ mole immobilized anti-albumin in 0.05 M sodium phosphate, 0.5 M NaCl, 0.1% gelatin, pH 7.0, was mixed with 7.6×10^{-5} μ mole 125 I labelled human albumin. 75 μ l of the immobilized anti-albumin (1.6×10^{-3} μ mole) was found to have bound 75% (5.7×10^{-5} μ mole) of the added albumin; 4. 25 μ l of sheep erythrocytes (25% in 0.05 M sodium phosphate, 0.9% NaCl, 0.05 M EDTA, 0.1% gelatin, pH 7.2) was mixed with 30 μ l 125 I labelled anti-sheep erythrocyte antibodies (15 μ g/ml). Approximately 2.8×10^{-4} μ mole IgG was found to have bound to the erythrocytes. The mixtures were incubated for 30 min at 37°C. In systems 1 and 2 the mixtures were incubated for 1 h at room temperature with 8.1×10^{-5} μ mole IgM, before the

pellets were centrifuged down and washed, and their radioactivity measured. The total error of the pipetting and washing steps, and the radioactivity measurements was 10%. Experiments were repeated four times, samples without additional IgM being run in parallel. The bound activity was calculated relative to the total activity added. The effect of the IgM was determined by comparing the samples with additional IgM with those without additional IgM. In systems 3 and 4 the pellets were washed and bound radioactivity determined, whereupon they were re-suspended in buffer before 8.1×10^{-5} μ mole IgM was added. After the mixtures had been incubated for 1 h at room temperature, and the pellets had been washed again, the ^{125}I activity of the pellets was measured. Significant differences in the radioactive measurements were defined as two standard deviations (SD), where $\text{SD} = \sqrt{n}$ and n = counts per minute (cpm). Experiments were repeated four times, samples without added IgM being always run simultaneously. The activity of the pellet before the incubation with IgM was set to 100%.

RESULTS

The IgM originating from rheumatoid serum was found to have RF activity (Table I), while normal IgM was RF negative. Neither albumin, C1q, nor C3a,b,c or d could be detected in the IgM. The RF IgM was found to contain IgA and IgG (<5%).

The precipitin curve obtained for the mixtures of human albumin and anti-albumin IgG is given in Figure 1. The point with the largest antigen excess (S) corresponds to the reaction mixture with the lowest albumin concentration analysed when the soluble system was used. Points 1,2 and 3 show the antigen/antibody ratio used for the immobilized systems 1,2 and 3.

The effect of isolated RF IgM on the soluble albumin anti-albumin system is shown in Table I. A significant increase in the amount of free albumin was observed both in electro-immuno assay and in single radial immunodiffusion, the increase obtained being the same with both techniques.

The anti-albumin IgG was pepsin digested. Gel electrophoresis and immunoelectrophoresis against anti-rabbit Fc γ and anti-rabbit Fab γ was used to control the digestion. No separation of the fragments was performed. The effect of RF IgM on albumin/pepsin digested anti-albumin IgG determined by electroimmuno assay is shown in Table II. To get the same height of the precipitin peaks for pepsin

digested and intact antibodies, twice as much of the pepsin-digested as of the intact antibodies was used. The significant increase of free antigen observed when intact antibodies were used was replaced by a significant decrease when pepsin digested antibodies were used.

To determine whether this treatment affected its ability to elicit an increased amount of free antigen, RF IgM was trypsin digested, gelelectrophoresis being used to monitor the digestion. RF IgM showed no RF activity after the digestion. Results given in Table III were obtained by electroimmuno assay, and show that the effect of RF IgM was less after trypsin digestion than before, indicating that the intact RF IgM molecule was responsible for the effect.

RF IgM labelled with ^{125}I was also added to the soluble albumin/anti-albumin system. Autoradiography showed IgM as a diffuse background covering the whole area within the precipitin peaks in the electroimmuno assay, and as diffuse rings with diameters smaller than the albumin/anti-albumin precipitates in the single radial immunodiffusion analysis. This meant that the RF IgM had not been incorporated into the precipitates formed.

RF IgM and normal IgM were both separated in IgM with, and IgM without affinity to albumin. Both RF IgM with

(55%) and without affinity to albumin had the same RF titres and caused similar increases in the amount of free albumin (Table IV). Neither the normal IgM with affinity (50%) to albumin, nor that without affinity to albumin, had any effect (Table IV). Thus the results showed that the effect of both RF and normal IgM was unrelated to their affinity to albumin.

The effect of RF IgM on immobilized antigen-antibody systems was also determined. First it was ascertained whether RF IgM could bind to the immobilized proteins, when it was found that 1-5% of the ^{125}I labelled RF IgM was bound. Furthermore, it was shown that the addition of RF IgM caused agglutination of the erythrocytes coated with antibodies. Table V shows that RF IgM had no detectable effect on the binding of antibody or antigen to the respective immobilized antigen and antibody when the unbound proteins were in excess. Table VI shows that additional RF IgM had no demonstrable effect in the immobilized systems, even when the excess of antigen or antibody had been removed before IgM was added. Interestingly, the differences in effect obtained with the soluble and immobilized systems were unrelated to differences in sensitivities. The amount of protein that had to be released in order to determine a significant change was: in system 1, 1.4×10^{-5} μmole IgG; in system 2, 7.6×10^{-6} μmole albumin; in system 3, 1.3×10^{-6} μmole albumin; and in system 4, 6.6×10^{-6} μmole IgG. No effect of RF IgM could be detected in these systems, not even when the incubation time with IgM was increased from 1 h up to overnight.

DISCUSSION

The antigen excess in the soluble system was so great, from nine up to twenty-four times equivalence, that no precipitation of antigen-antibody complexes occurred in the reaction mixtures. The difference in amount of free antigen between standard and sample series was thus a difference in the amount of unbound antigen, or a difference in the amount of or the composition of the soluble antigen-antibody complexes that were applied to the gel plates or formed in the gel.

The trypsin digested RF IgM, unlike the undigested protein, showed no RF activity and had less effect on the soluble antigen-antibody complexes. This indicated that RF's ability to dissociate soluble antigen-antibody complexes was dependent on the RF activity which was in turn dependent on the intactness of the molecule.

It is well known that RF reacts with the Fc part of IgG (5). Here, RF IgM was unable to dissociate soluble antigen-antibody complexes once the Fc γ part of the antibody had been split off. Therefore the mechanism responsible for the dissociation involved an interaction between RF IgM and the Fc γ of the antibody in the complexes.

Czop and Nussenzweig (4) showed that precipitates of bovine serum albumin and anti-bovine serum albumin were solubilized by Fab γ fragments directed against the

antibodies, but were stabilized by $F(ab)_2\gamma$ of the same antibodies. Their results agree to some extent with those obtained here, if RF is assumed to be polyvalent when it reacts with the immobilized system. They also partially agree with reports that RF IgM precipitates antigen-antibody complexes formed in slight antigen excess (16, 19), but they do not agree with our finding that RF IgM was not incorporated into the precipitates. Here it was found that dissociation of the soluble complexes formed in gross antigen excess does occur when RF is present. This effect is noteworthy, as it may be more significant biologically than the effect on precipitates, especially where the relationship between circulating immune complexes and its relation to disease is concerned.

Since RF IgM elicited the same amount of free antigen, whether the albumin/anti-albumin system was analysed by electroimmuno assay or by single radial immunodiffusion, the electrical forces applied in the electroimmuno assay analyses clearly did not influence the result obtained. Furthermore, these results enhance the reproducibility of the effect.

Normal IgM and macroglobulinaemic IgM have been shown to decrease the amount of free antigen in mixtures of antigen in excess and antibody, regardless of the type of antigen or species origin of antibody (2). These results

were confirmed here by the fact that normal IgM with affinity for albumin was found to have about the same effect as IgM without affinity. The explanation of the effect of normal IgM might be that, since IgM is hydrophobic molecules, it reacts with immune complexes, owing to an increase in hydrophobicity when antigen reacts with antibody. In the case of RF IgM this hydrophobic binding is overcome by the higher affinity of RF to Fc_γ of the antibody in the complexes.

A dissociation of immune complexes on cell surfaces may be a disadvantage for the elimination of immune complexes by phagocytizing cells (8,20). The immobilized systems were used to clarify what occurs when antigen-antibody complexes are firmly bound to cell surfaces, or when the antigens are the surface antigens on a cell. These experiments could also give information whether the primary antigen-antibody bindings were affected by RF IgM. The results show that if RF affected such complexes at all, it affected them less than it did soluble complexes. These differences may perhaps be explainable by variations in steric conditions, for instance, the comparative bulkiness of Sepharose particles and erythrocytes, by conformational changes caused by immobilization, or by non-optimum distances between immobilized proteins, or by the fact that it is only the secondary bindings between antigen-antibody complexes that RF IgM is able to dissociate.

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TABLE I. Effect of RF IgM on soluble albumin/rabbit
anti-albumin system. Electroimmuno assay (EIA)
and single radial immunodiffusion (SRID)

Method of analysis	Increase in amount of free albumin (μ mole)	Standard deviation (μ mole)	95% confidence interval (μ mole)	RF titer
EIA	1.1×10^{-5}	6.7×10^{-6}	0.40×10^{-5}	1:320
SRID	1.0×10^{-5}	1.8×10^{-6}	0.11×10^{-5}	1:320

TABLE II. Effect of RF IgM on the soluble albumin/
 rabbit anti-albumin system before and after
 the anti-albumin IgG had been pepsin digested.

Anti-albumin	Change in amount of free albumin (μ mole)	95% confidence interval (μ mole)
Pepsin	-1.1×10^{-5}	-0.30×10^{-5}
Intact	1.1×10^{-5}	0.40×10^{-5}

TABLE III. Effect of trypsin digested RF IgM on the
soluble anti-albumin system

RF IgM	Increase in amount of free albumin (μ mole)	95% confidence interval (μ mole)	RF activity
Trypsin	0.47×10^{-5}	0.14×10^{-5}	Neg
Intact	1.1×10^{-5}	0.40×10^{-5}	1:320

TABLE IV.

The effect, on the soluble albumin/anti-albumin system, of IgM with affinity for albumin compared with that of IgM without affinity for albumin, and comparison of RF and normal IgM.

IgM	Affinity for albumin	Change in amount of free albumin (μ mole)	95% confidence interval (μ mole)	RF titer
RF	Yes	0.82×10^{-5}	0.44×10^{-5}	1:80
RF	No	0.64×10^{-5}	0.35×10^{-5}	1:80
Normal	Yes	-0.11×10^{-5}	-0.44×10^{-5}	Neg
Normal	No	0.41×10^{-5}	0.52×10^{-5}	Neg

TABLE V. Effect of RF IgM on the binding of antibody (system 1) and antigen (system 2) to immobilized antigen (system 1) and antibody (system 2) when the free proteins were in excess and labelled with ^{125}I . System 1 corresponds to point 1 and system 2 to point 2 in Figure 1.

System	IgM	Radioactivity in the pellet after incubation with IgM as a percentage of the total activity added ± 2 SD
1	RF	52.9 ± 2.9
	None	52.3 ± 2.8
2	RF	63.0 ± 4.1
	None	60.6 ± 4.1

TABLE VI. Effect of RF IgM on the solubilization of antigen (= albumin in system 3) or antibody (= anti-sheep erythrocyte IgG in system 4) when the excess of ^{125}I labelled antigen or ^{125}I labelled antibody was removed before IgM was added. The immobilized phase contained anti-albumin in system 3 and surface antigen on sheep erythrocytes in system 4. System 3 corresponds to point 3 in Figure 1.

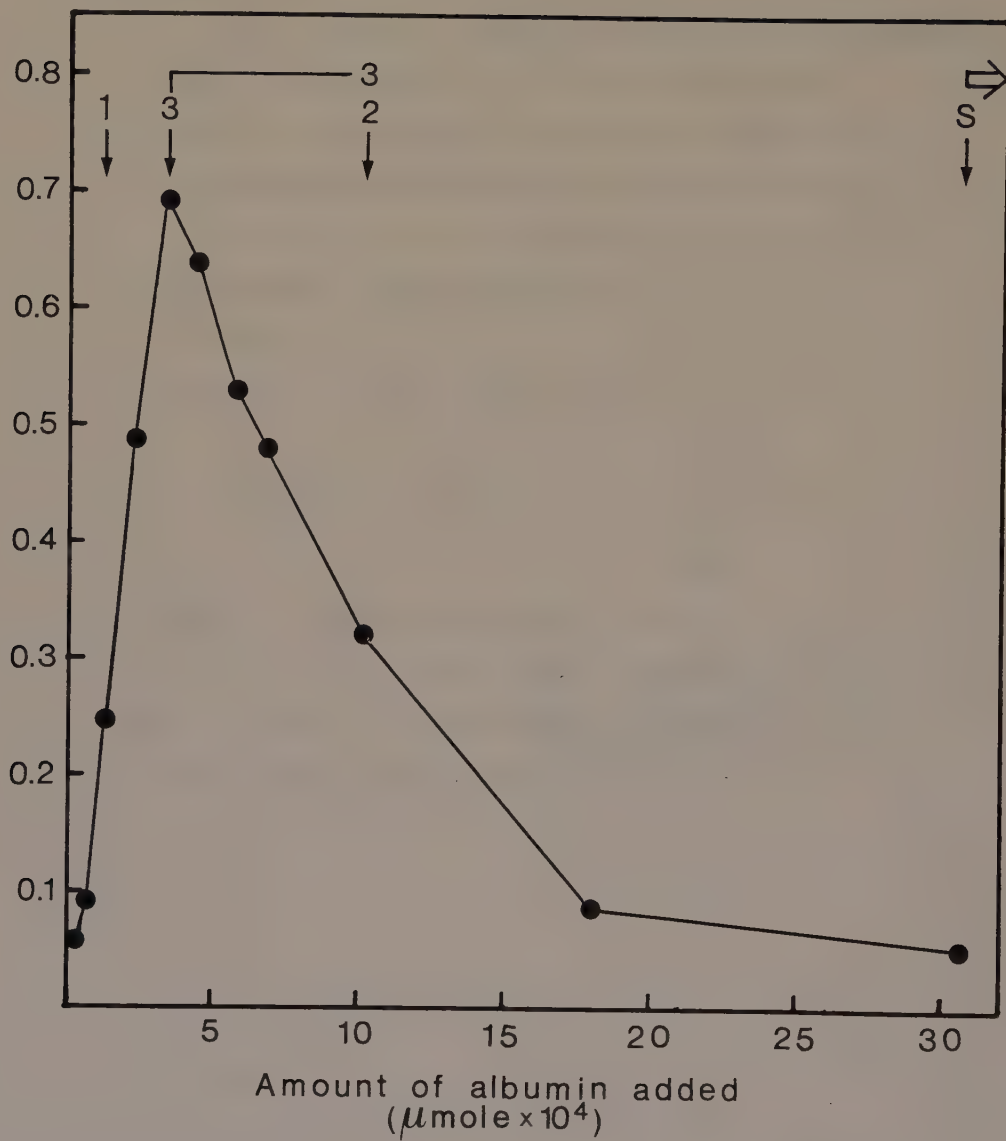
System	IgM	Radioactivity in the pellet after incubation with IgM in per cent of the pellet activity before addition of IgM $\pm$ 2 SD
3	RF	96.4 $\pm$ 2.3
	None	96.8 $\pm$ 2.5
4	RF	89.8 $\pm$ 2.5
	None	88.4 $\pm$ 2.5

LEGEND

Figure 1. Quantitative precipitin curve obtained when an increasing amounts of human albumin were added to 2.6×10^{-3} μ mole of anti-human albumin IgG. The arrows indicate the antigen/antibody ratios for

1. immobilized albumin/free anti-albumin.
System 1.
2. free albumin/immobilized anti-albumin.
System 2.
3. albumin/immobilized anti-albumin.
System 3.
- S. soluble albumin/anti-albumin system. The unfilled arrow indicates that the soluble system was analysed in an antigen excess equal to or greater than that at point S.

Absorbancy of the
dissolved precipitates
at 280nm



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Isolation and Characterization of Intermediate Complexes and Other Components with Common Antigenic Determinants

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Hansson, U.-B., Uesson, M. & Alkner, U. Isolation and Characterization of Intermediate Complexes and Other Components with Common Antigenic Determinants. *Scand. J. Immunol.* 13, 57–66, 1981.

Fractions with affinity for heat-denatured human IgG (HDG) were isolated from four sera that contained intermediate complexes (IC). These IC fractions contained part of the 7S IgG, all IC, and part of the rapidly sedimenting complexes (RC) found in the sera. The IC consisted of IgG1 or IgG3 and the RC of IgG and IgM with kappa and lambda light chains. The IgG in the IC fractions contained an abnormally large amount of neuraminic acid. No correlation between IgG subclass or content of neuraminic acid and complex formation was found. There were indications that the formation of IC was not only the result of a self-association of IgG molecules with anti- γ -globulin activity. Specific rabbit antisera were prepared against two of the IC fractions. Affinity chromatography with immobilized IgG and F(ab')₂ γ from these antisera confirmed the presence of common antigenic determinants in the sera. These determinants occurred mainly in 7S components in one individual, in IC in one and in RC in another. Only a minor part of the serum components with affinity for HDG contained the determinants. RF activity was found in the components that lacked and in those that contained the common antigenic determinants.

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Intermediate complexes (IC) have been demonstrated in sera of patients with rheumatoid arthritis (RA) and of those with hypergammaglobulinaemic purpura [24, 35, 40].

The IC in three RA sera were formed by self-association of IgG rheumatoid factors (IgG RF) [36], and the IC in one non-RA serum were formed due to IgG aggregation rather than to an antigen–antibody reaction [16].

IgG in IC differs from normal IgG. A restriction to subclass 1 [15] and an abnormally large content of neuraminic acid [17] have been reported. Differences between IgG RF and normal IgG have also been demonstrated. IgG1 is more dominant in IgG RF than would be expected from the subclass distribution in normal human IgG [2]. The fragments obtained on papain hydrolysis of IgG RF and normal IgG are different [46], and so are the circular di-

chromism spectra of IgG from RA and non-RA patients [21].

Unique antigenic determinants present in a group of molecules with similar function have been referred to as cross-idiotypic [22]. Such determinants occur in human monoclonal IgM RF [23] and are shared by monoclonal and polyclonal IgM RF and by monoclonal IgG RF and polyclonal IgM RF [5]. Attempts have been made to prepare antibodies to human polyclonal IgG RF [20] and human IgG dimers from RA serum [36], but to our knowledge no reports are available about common antigenic determinants in polyclonal IgG RF or IgG complexes with RF activity.

In this study immunoglobulin complexes were isolated from four sera that contained intermediate complexes. The isolated fractions were characterized with regard to RF activity,

dissociation and association of the complexes, IgG subclass distribution, and neuraminic acid content. Rabbit antibodies were then prepared which reacted specifically with unique antigenic determinants in some 7S components, some intermediate complexes, and some rapidly sedimenting complexes.

MATERIALS AND METHODS

Sera. Sera from four patients with rheumatoid arthritis (RA sera; OS and ED) or hypergammaglobulinaemic purpura (non-RA sera; MG and BL) were analysed. Three of the patients (OS, ED and BL) also had a hyperviscosity syndrome, and one (ED) also had hypergammaglobulinaemic purpura. Normal sera from registered blood donors were used as controls. The sera had been stored at -20°C .

Antisera against normal human serum proteins. Anti-kappa, anti-lambda, anti-Fab γ , anti-Fc γ , and anti-C1q were from Behringwerke AG, Marburg, West Germany. Anti-whole human serum was from DAKO-immunoglobulins Ltd, Copenhagen, Denmark, or from Hyland, Division Travenol Labs. Inc., Los Angeles, Calif., USA. Anti- α -lipoproteins and anti- β -lipoproteins were from the Department of Clinical Chemistry, University of Lund, Lund, Sweden. Anti-IgG1, 2, 3 and 4 were from Nordic Immunol. Labs., Tillburg, The Netherlands. Anti-IgG, anti-IgM and anti-IgA were prepared in our laboratory by hyperimmunizing with the purified immunogen in complete Freund's adjuvant and were appropriately absorbed. All antisera except the IgG sub-class-specific ones (swine) originated from rabbits.

Isolation of human and rabbit IgG. Human IgG was a commercial preparation for human use (AB Kabi, Stockholm, Sweden). 7S IgG was obtained from this preparation by gel filtration on Sephadex G-200 in 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. Human myeloma 7S IgG of subclass 1, 2 and 3 was isolated as described earlier [12].

Rabbit IgG was prepared from serum by salting out with 1.84 M $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C . The washed precipitates were dissolved and dialysed against 0.16 M NaCl. The non-specific rabbit IgG was then applied to a DEAE-cellulose column, from which the IgG was eluted with 0.02 M sodium phosphate, pH 8.0.

Isolation of IC fractions. Sepharose 6B containing 500 mg immobilized [4] heat-denatured (63°C , 30 min) human IgG (HDG) was used. Sequential elution was made with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1; 1 M NaCl; and 0.2 M 2-aminobutane, 0.075 M Tris, 10^{-3} M CaCl_2 , pH 11.0. The eluates were concentrated by vacuum dialysis in collodion bags against 0.16 M NaCl, 0.1 M glycine, pH 7.0, to avoid non-specific aggregation and precipitation [11] IC fraction referred in all cases except BL (pool of delayed Tris fractions) to the 2-aminobutane pool. The maximum binding capacity of the columns was never exceeded.

Preparation of specific antiserum against antigenic determinants in the IC fraction. Fractions from BL and ED were used to hyperimmunize ten rabbits

each. 1 mg immunogen dissolved in 0.5 ml 0.16 M NaCl, 0.1 M glycine, pH 7.0, was mixed with 0.5 ml complete Freund's adjuvant (Difco, Detroit, Mich., USA) and was injected at several sites behind the scapulae of each rabbit. The rabbits were given booster doses in the same way when required. A part of each antiserum pool was absorbed with normal human serum. After this absorption four of the antisera contained antibodies that precipitated with the immunogen. Two were used for preliminary experiments only, and the other two, which had higher titres, were used in the study. The antiserum a-BL was absorbed on a larger scale by dropwise addition of normal human serum. The final absorption of a-BL and the sole absorption of a-ED were performed with affinity chromatography to immobilized normal human serum coupled [4] to Sepharose CL-4B. IgG was isolated from the antisera before the antibodies were applied to the immunosorbent columns. The antibody fraction that lacked affinity to the ligand was eluted with 0.075 M Tris, 0.075 M NaCl, 10^{-3} M CaCl_2 , pH 8.1, and concentrated by vacuum dialysis as described under IC fractions. The specificity of the absorbed antibodies was assessed by double immunodiffusion against normal human IgG, normal human serum, and the immunogens over a wide range of concentrations (0.02–400 g protein/l). Precipitates were obtained only with the immunogens, and BL could be diluted to at least 0.02 g/l and ED to at least 0.08 g/l without disappearance of the precipitation with undiluted a-BL and a-ED, respectively.

Enzymatic digestions. Pepsin digestion was performed as described by Turner *et al.* [44] and papain digestion according to Porter [37]. The digestions were checked by ultracentrifugal analysis and by immunoelectrophoresis against anti-Fab γ and anti-Fc γ . After papain digestion more than 95% of the material in OS, BL and MG had a mean sedimentation coefficient (s_{20w}) of 3.35S–3.85S (± 0.10) (depending on the protein concentration). The IC fraction ED contained 25% IgM, and a corresponding amount of 5S components was found on ultracentrifugation of the papain-digested ED. The amounts of Fab γ and Fc γ were estimated by measuring the density of the agarose gel electrophoretic bands by a densitometer.

Neuraminidase treatment was performed in 0.05 M NaAc, 0.05 M CaCl_2 , pH 5.5. Four units of neuraminidase was added per milligram protein, and the sample was incubated at 37°C overnight. The enzyme reaction was inhibited by dialysis against 0.05 M Tris, 0.01 M EDTA, pH 8.2 [1]. The effectiveness of the reaction was checked with agarose gel electrophoresis.

Isolation of F(ab') $_2$ Y fragments. Commercial normal human IgG, IgG from a-BL, a-ED and IgG from non-immunized rabbits were pepsin-digested, applied to a column of Sephadex G-200, and eluted with 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. The appropriate components were selected. The absence of undigested 7S IgG was checked by analytical ultracentrifugation against a standard human 7S IgG preparation and by immunoelectrophoresis. In these experiments the protein concentration in the F(ab') $_2$ Y fractions was 10 g/l, and control experiments showed that 2% IgG in the samples would be detected.

Isolation of serum proteins with affinity to immobilized IgG or F(ab') $_2$ Y. The IgG or the F(ab') $_2$ Y was coupled

[4] to Sepharose 6B. Elution and concentration of the fractions was done as described under isolation of IC fractions.

Ultracentrifugal analysis. Ultracentrifugal analysis was done with an An-D rotor at 59,780 (259,700 g) rpm in a Beckman Analytical Ultracentrifuge Model E with Schlieren optics. The temperature was 20°C, and the sample was dissolved in 0.16 M NaCl, 0.1 M glycine. The total protein concentration was always below 20 g/l, and no correction was made for the Johnston-Ogston effect. s_{20W} values were obtained by making the usual correction for density and viscosity using a $\bar{v}$ value of 0.723 [31]. s_{20W}^0 values were determined as described previously [12].

The s-rate distribution of the components in the samples was determined as described previously [10].

In experiments determining the effect of additional IgG on the distribution of components in the IC fractions an amount of IgG equal to that originally present in the IC fraction was added.

Qualitative and quantitative protein determinations. The protein concentration in all eluates and fractions was assayed by the light absorbancy at 280 nm ($E_{1\%}^{1cm}$ 280nm of 12.5) [29]. The serum protein concentrations were determined according to Lowry *et al.* [27]. Electroimmunoassay was used to quantify Clq and single immunodiffusion to quantify IgG and IgM. Owing to variation of the diffusion rate between 7S IgG and IgG-IgG complexes, the errors in the determination of the IgG concentration was $\pm 5\%$. IgM was quantified in the reduced samples (0.1M β -mercaptoethanol, 4°C, overnight) with an error of less than $\pm 3\%$. The errors were expressed as maximum deviation from the mean.

Single immunodiffusion was performed according to Mancini *et al.* [28], double immunodiffusion according to Ouchterlony [34], immunoelectrophoresis according to Scheidegger [41] and electroimmunoassay according to Laurell [26]. These analyses and the agarose gel electrophoresis were run in 0.8% agarose gel in 0.075 M barbital buffer, 2 mM calcium lactate, pH 8.6. The calcium lactate was excluded in the Clq analyses.

RF activity. The RF activity of sera and IC fractions was determined with the acryl fixation test according to Winblad [48]. The RF activity of fractions isolated from the immobilized F(ab')₂γ fragments of a-BL and a-ED was determined with a modified Waaler-Rose test [13] in which EDTA, glycine and NaCl were used to liberate hidden rheumatoid factors and to inactivate the complement. The RF activity in the a-BL and a-ED antibodies was determined with a latex fixation test, modified in the corresponding manner [13]. The reaction was always considered positive when the titre was at least 60.

Major IgG subclass. The major IgG subclass was established by high-voltage electrophoresis [7] and immunoelectrophoresis on papain-digested samples [39]. Double immunodiffusion and immunoelectrophoresis against IgG subclass-specific antisera were used to check whether the specificity of these antisera corresponded to the results obtained on estimation of the major subclass and to ascertain the presence or absence of minor subclasses.

Neuraminic acid content. The neuraminic acid content was determined according to Warren [45]. The error

was $\pm 7\%$. In the IC fraction ED 25% (w/w) of the proteins were IgM. The neuraminic acid content per mole of 7S IgG (x) was in this case calculated according to the formula $0.75 \times x + 0.25 \times 6$ = moles of neuraminic acid per mole 7S component (assuming six residues of neuraminic acid per 7S unit of IgM [30]).

RESULTS

The distribution of intermediate (IC) and rapidly sedimenting (RC) complexes in the four sera is given in Table I. The two RA sera, OS

TABLE I. Distribution of intermediate and rapidly sedimenting complexes in the sera (% of total protein)

Serum	Intermediate complexes		Rapidly sedimenting complexes	
	9S	>9S < 19S	19S	> 19S
OS*	7	20	0	2
ED*	15	3	0	27
MG	0	6	2	0
BL	8	0	3	0

* Sera from the patients with rheumatoid arthritis and a positive rheumatoid factor titre.

and ED, contained IC with two s-rates and RC, whereas the two non-RA sera contained IC with only one s-rate.

To isolate the IC and the RC, the sera were applied to columns containing immobilized heat-denatured human IgG (HDG). Fractions without affinity were invariably eluted as a large single peak in Tris buffer, pH 8.1. Besides this major peak BL showed a second peak. 40% of the proteins in serum BL were found in this delayed Tris buffer fraction. No proteins were in any case eluted with 1 M NaCl. 30% of the proteins in OS, 26% of the proteins in ED, 22% of those in serum MG, and 5% of those in normal serum had a high affinity for the label and were eluted with the third elution buffer, the 2-aminobutane buffer, pH 11.0. The proteins in the fractions with affinity—that is, the 2-aminobutane fractions, and, in the case of BL, the delayed Tris buffer fractions—migrated in the γ -region on agarose gel electrophoresis. They were RF-positive (titre ≥ 60), although

TABLE II. Distribution of components in the fractions with affinity to heat-denatured human IgG (HDG) (% of total protein)

Sample	Obtained distribution*				Predicted distribution†			
	7S	9S	>9S≤19S	>19S	7S	9S	>9S≤19S	>19S
OS	3	5	83	9	4	25	65	6
ED	5	42	16	37	0	59	12	29
MG	82	0	16	2	68	0	32	0
BL	60	40	0	0	73	20	7	0

* Obtained by ultracentrifugal analysis of the fractions with affinity to HDG, i.e. the IC fractions.

† Obtained by ultracentrifugal analyses of serum and the corresponding fractions without affinity to HDG. The predicted distribution was obtained by subtracting the amount of the different components in the fractions without affinity to HDG from the amount of the corresponding components in the serum.

only the RA sera OS and ED had shown RF activity.

Ultracentrifugal analyses showed that IC existed only in the fractions with affinity for HDG. The s-rate distribution of components in the IC fractions is given in Table II. Besides IC and RC, the fractions from the RA sera contained traces and those from the non-RA sera large amounts of 7S components.

The changes, if any, in 'composition' of the complexes due to isolation were determined from the s-rate distribution in serum and the isolated fractions by comparing the obtained and the predicted distributions (Table II). On isolation some of the complexes aggregated in OS and ED but dissociated in MG and BL.

All the IC fractions contained both kappa and lambda light chains. Not only IgG and IgM but also α - and β -lipoproteins (1%) and Clq were found. The IC in three of the fractions consisted mainly of IgG; that is, more than 90% of the proteins were IgG and less than 4% was IgM. The fourth fraction, ED, contained 25% IgM besides the IgG, so that no definite conclusion about the composition of the IC could be drawn. In all the cases except BL, the major IgG was of subclass 1, with traces of IgG2 and IgG3. BL contained IgG3 and traces

of IgG1 and 2. The Clq content in the sera and IC fractions is given in Table III. The IC fractions OS and MG contained almost all, and the fractions ED and BL only part of, the Clq found in serum.

The effect of additional 7S components of normal human IgG or myeloma IgG1, 2 and 3 on the complex formation was also assessed. Ultracentrifugal analysis of the IC fractions showed that after the additions the amount of IC increased by more than 25% and the amount of 7S components decreased by more than 10%. In the non-RA samples the opposite occurred. The amount of IC decreased by more than 25% at the same time as the amount of 7S components increased by more than 10%. There was no correlation between the observed effect and the subgroup of the added IgG.

Determinations were made also of the affinity for immobilized normal human 7S IgG and for F(ab')₂γ fragments thereof. No proteins were ever found in the NaCl fractions. When serum BL was applied to the F(ab')₂γ column, no proteins were detected as a delayed Tris fraction or in the 2-aminobutane buffer. But when intact IgG was used, 10% of the applied proteins were found in the 2-aminobutane buffer and 47% as a delayed Tris fraction. Of the proteins in serum ED 2% had affinity for F(ab')₂γ and 24% for the intact IgG.

The neuraminic acid content was abnormally high in all the IC fractions. Normal human IgG contained 0.7, OS 3.2, ED 2.7, MG 2.4, and BL 2.7 mole of neuraminic acid per mole IgG.

Ultracentrifugal analysis showed that neither the complex formation nor the reactivity with

TABLE III. Clq content in sera and intermediate complex (IC) fractions (g/l)

	OS	ED	MG	BL
Serum	0.38	0.40	0.17	0.77
IC fraction	0.32	0.10	0.18	0.11

HDG was affected when the fractions were treated with neuraminidase.

The IC fractions were papain-digested. They were re-applied to the immobilized HDG after the effectiveness of the hydrolysis had been checked. The results are given in Table IV.

TABLE IV. Amount of Fc γ and Fab γ fragments that had affinity for heat-denatured human IgG when the intermediate complex fractions had been papain-digested (% of total amount of the same fragment)

	OS	ED	MG	BL
Fab γ	70	50	75	25
Fc γ	25	80	25	95

Since no proteins were eluted with NaCl or as a delayed Tris buffer fraction, proteins with affinity were eluted with 2-aminobutane buffer and fractions without affinity as major Tris fractions. A large part of the Fab γ fragments in the IC fractions had no affinity for HDG. Various amounts of the Fc γ fragments were found in fractions without affinity.

Antisera were thereafter prepared against the IC fractions BL and ED. The absorbed antisera (a-BL and a-ED) precipitated proteins in the immunogenic fraction but not in other fractions isolated from the corresponding serum; that is, the precipitin reaction was restricted to the proteins with affinity for immobilized HDG. Native or heat-denatured normal human IgG did not precipitate a-BL and did not inhibit the precipitation between the immunogenic BL fraction and a-BL. The antisera showed no RF activity. The IC fractions did not precipitate rabbit γ -globulins isolated from non-immunized rabbits.

IgG from a-BL and a-ED was isolated and immobilized. 1% of the proteins in normal human serum had affinity for these labels (Table V). More than 10% of the proteins in all the sera, except ED, had affinity for both labels. More proteins in ED had affinity for the a-BL than for the a-ED IgG. Immunodiffusion showed that the proteins that had affinity for immobilized a-BL were mainly IgG, whereas those with affinity for a-ED were IgG and IgM.

F(ab') $_2\gamma$ fragments prepared from an IgG isolated from non-immunized rabbits were thereafter immobilized, and the affinity to this

TABLE V. Amount of serum proteins with affinity for immobilized IgG of a-BL and a-ED (% of total serum protein)

Serum	a-BL IgG	a-ED IgG
NS	1	1
OS	12	13
ED	18	4
MG	12	12
BL	40	27

label was determined. Proteins in normal human serum or serum BL had no affinity, whereas 1% of the proteins in serum OS and 2% of the proteins in serum ED had affinity.

F(ab') $_2\gamma$ fragments prepared from the a-BL and a-ED IgG were then immobilized. The results obtained when the affinity for these labels was determined are given in Table VI.

TABLE VI. Amount of serum proteins with affinity for immobilized F(ab') $_2\gamma$ of a-BL and a-ED (% of total serum proteins)

Serum	a-BL F(ab') $_2\gamma$	a-ED F(ab') $_2\gamma$
NS	0.3	0.4
OS	3.3	4.0
ED	10.1	6.5
BL	9.8	1.3

Serum MG could not be analysed because of scarcity of material. Except for the ED/a-ED pair, the amount of proteins with affinity for F(ab') $_2\gamma$ was lower than the amount that had affinity for the intact antibodies (compare with Table V). For serum ED the amount with affinity for the a-ED F(ab') $_2\gamma$ was larger than that with affinity for the a-ED IgG.

The s-rate distribution of components in the fractions that had affinity for the F(ab') $_2\gamma$ fragments of a-BL and a-ED is given in Table VII. For one and the same serum the proteins that had affinity for the a-BL and a-ED fragments, respectively, had a remarkably similar s-rate distribution. Both the OS fractions thus contained mainly IC, both the ED fractions mainly RC, and both the BL fractions mainly 7S components. The fractions from normal serum contained mainly 7S components. Ultracentrifugal analysis of the fractions without affinity for the F(ab') $_2\gamma$ fragments confirmed

TABLE VII. S-rate distribution of the serum components with affinity for immobilized $F(ab')_2\gamma$ of a-BL and a-ED (% of total protein)

Sample with affinity for:		7S	9S < 19S	> 19S
a-BL $F(ab')_2\gamma$	a-ED $F(ab')_2\gamma$			
OS		42	58	0
	OS	31	69	0
ED		0	0	100
	ED	0	0	100
BL		100	0	0
	BL	100	0	0

the data showing that only a part of the 7S, IC, and RC components in the sera had affinity for the column ligand.

There was no relation between RF activity and affinity for the immobilized a-BL and a-ED $F(ab')_2\gamma$ fragments. RF activity was found both in fractions with and in fractions without affinity for these fragments. None of the BL fractions had any RF activity, which should be compared with the low RF activity found in BL fractions with affinity for HDG.

Agarose gel electrophoresis showed that the proteins in the fractions with affinity for the $F(ab')_2\gamma$ fragments of a-ED and a-BL migrated in the γ -zone. The precipitin arcs obtained on immunoelectrophoresis against anti-whole human serum were without exception found in the same region. Single and double immunodiffusion showed that the fractions from BL contained mainly IgG and small amounts of IgM (1%). The fractions from OS and ED contained mainly IgG, but also IgM (12–31%). All fractions contained both kappa and lambda light chains. All fractions, except BL from a-ED $F(ab')_2\gamma$, contained Clq, as did all the fractions without affinity.

To determine what part of the IgG molecule had affinity for a-BL, the components in serum BL with affinity for the a-BL $F(ab')_2\gamma$ were pepsin-digested, and the $F(ab')_2\gamma$ components were isolated and run again on the column. All $F(ab')_2\gamma$ had affinity; that is, the antigenic determinants were located in the $F(ab')_2\gamma$ fragments of the molecules.

DISCUSSION

The nature of the solvents may affect the poly-

merization of anti- γ -globulins [36]. To facilitate a comparison of the isolated fractions, the same solvents and immunosorbent technique were used to isolate IC and other serum components from all the sera.

We found no evidence that the affinity for the immobilized proteins depended on non-specific interactions. The small amount of components in normal human serum with affinity for, for example, HDG was compatible with the demonstrated occurrence of anti-globulins also in normal human sera [14]. Our results also confirmed those obtained by Nardella & Mannik [32] showing that the non-immunospecific interactions of IgG and IgG-agarose are weak or non-existent at pHs above 4 and ionic strengths corresponding to an NaCl molarity of 0.5.

The IC fractions isolated from the non-RA sera contained a large amount, and those from the RA sera a small amount, of 7S components with affinity for HDG. The RA sample ED contained more than 5% of the proteins as IgM, and 2% of the proteins in this sample had affinity for $F(ab')_2\gamma$ of normal IgG. Self-association of IgG molecules with anti- γ -globulin activity might be inhibited by IgM (>5%) and should not be expected when the anti- γ -globulin molecules show anti-Fab γ activity [36]. The existence of 7S IgG in fraction ED could thus be explained by an anti-Fab γ activity or a high content of IgM. The two non-RA samples, which contained large amounts of non-aggregated IgG with affinity for HDG, contained less than 4% IgM and no anti- γ -globulins with anti-Fab γ activity. This indicated mechanisms other than self-association for the complex formation.

All of the monomeric IgG and the IC in

serum BL had affinity for HDG when columns containing HDG bound to Sepharose were used. No agglutination was observed, however, when serum was added to latex particles coated with an identical HDG. One explanation of these differences could be that the binding sites of the Fab γ with affinity for HDG were occupied in the serum but became free on the gel filtration in the chromatography column. This could also explain why some Fab γ fragments obtained after papain digestion had a higher affinity for immobilized HDG than had the intact IgG molecules and IC.

The IC in all the sera had affinity for HDG. The isolated complexes were, however, affected in different ways when native human IgG was added. The IC from the non-RA sera dissociated and the IC from the RA sera combined with the added IgG. Differences in reactivity thus occurred among groups of sera related to different diagnoses, as was also suggested by Halla *et al.* [9]. Differences in affinity between Fab γ and Fc γ within the complexes isolated from sera from individuals with the same diagnosis might, however, also occur. The interactions between the Fab γ and Fc γ in our IC fractions from the RA patients were weak or non-existent, but they were relatively high in the IC fractions from the RA patients studied by Pope *et al.* [36].

After the IC fractions had been papain-digested, Fab γ fragments without affinity for HDG were found in all the fractions. No intact IgG molecules were detected in the fractions. IgG has two identical Fab parts [43]. The complexes with affinity for HDG therefore contained more than one population of IgG molecules; one population contained Fab γ with affinity for HDG and the other contained Fab γ without such affinity.

Clq has affinity for immune complexes greater than 20S, for aggregated human IgG, for rheumatoid factors, and for aggregates composed of RF and soluble complexes [18, 42, 49, 50]. Most of the Clq was, as expected, recovered in those fractions that had affinity for HDG. However, part of the Clq in the ED serum had no affinity for HDG or those complexes that reacted with HDG. Clq could thus in this case be divided into subpopulations with different affinities. One part of this Clq was thus similar to that described by Chapuis *et al.* [3]. The Clq studied by them origin-

ated from an individual with severe lupus-like syndrome, and it also lacked ability to bind to Sepharose-IgG. Some Fc γ fragments from the papain-digested IC fractions were found in fractions with affinity for HDG. One plausible explanation of this was that Clq that had bound to HDG also combined with Fc γ in the applied fractions because Clq has several binding sites for Fc γ [38]. Any specific interaction between Fab γ and Fc γ was excluded because the monovalent Fab γ that bound HDG could not at the same time bind the Fc γ fragments.

As reported by others [2, 15], we also found that IgG1 dominated in three of our IC fractions. The effect of additional IgG on the dissociation/association of the complexes was not related to the subclass of the added IgG. The implication of the subgroup restriction and its relation to the complex formation therefore remained unclear.

The IgG in the IC fractions contained more neuraminic acid per mole than normal IgG. The same result has been obtained previously for the IC from a patient with RA [17]. The reassociation of these IC required the presence of neuraminic acid [17]. This was not the case for the IC studied here. Our proteins did not contain cryo-IgG, and they were not myeloma IgG—proteins that have been shown to contain an abnormal content of neuraminic acid [30, 51]. The significance of the high content of neuraminic acid in our samples thus remained unclear.

Complete Freund's adjuvant was used for the immunizations of rabbits with the IC fractions. The adjuvant effect is mediated by a peptidoglycolipid of *Mycobacterium tuberculosis* [47]. The injection of lipopolysaccharides might, in mice, elicit the production of rheumatoid factors that cross-react with human IgG [19]. The antisera obtained here did not react with latex particles coated with HDG or with normal IgG. We therefore had no reason to assume that our antisera reacted with the immunogenic IC fractions because of an anti-IgG activity of the antibodies.

The amount of proteins with affinity for the intact antibodies was much larger than that with affinity for the F(ab') γ of the same antibodies. Intact antibodies could therefore not be used to isolate components containing the common antigenic determinants. This was so even when the antisera had been absorbed on

immunosorbent columns and thus did not contain immune complexes due to overabsorption.

A small amount of proteins in all the sera had affinity for $F(ab')_2\gamma$ from non-immunized rabbits. Since this reactivity could partly be dependent on secondary reaction between complexes bound to $F(ab')_2\gamma$ and RF active complexes in solution, the absolute amount of proteins with $F(ab')_2\gamma$ affinity could not be calculated. But, because the amount that reacted with the non-specific $F(ab')_2\gamma$ was so small compared with that with affinity for the $F(ab')_2\gamma$ from the specific antibodies, the possibility that the reaction with the $F(ab')_2\gamma$ of the specific antisera depended on 'non-specific interactions' could be eliminated.

Clq was detected in all the fractions that lacked affinity for the immobilized $F(ab')_2\gamma$ from the specific antibodies, but not in all the fractions that had affinity for this label. This showed that Clq was not the antigen and that the combination with the specific antibodies did not occur through Clq.

Not only components in the sera from which the immunogenic molecules originated but also components in the other IC sera had affinity for the $F(ab')_2\gamma$ from a-ED and a-BL. It was interesting to note that the components with this affinity in serum ED were mainly larger than 19S (RC), in serum OS mainly of intermediate size (IC), and in serum BL mainly of the 7S type. This was true whether $F(ab')_2\gamma$ from a-ED or from a-BL was used as immunosorbent. Only a part of the RC, IC or 7S components found in the sera had the above-mentioned affinities. The actual antigen(s) was thus only exposed in some of the RC, IC or 7S molecules. The occurrence of this antigen was related to components of a special 'size' in one person but not to the 'size' of the components in another person.

The presence of immunogenic determinants in the $F(ab')_2\gamma$ part has been used as a criterion of idiotypic specificity [8], and such determinants also often reflect the specificity of antibodies [33]. Cross-idiotypic determinants have recently been described on polyclonal IgM RF and monoclonal IgG RF [5]. In the latter case $F(ab')_2\gamma$ fragments of the anti-idiotypic antibodies blocked the Waler-Rose reaction of the RF active molecules, which was taken as an indication that the antisera were directed against determinants in the antigen-binding

site of the RF [5]. It was shown that the $F(ab')_2\gamma$ fragments from BL were those that had affinity for the specific antibodies, but we found no relation between RF activity and reactivity with the specific antisera. Both fractions with and fractions without affinity for the $F(ab')_2\gamma$ of the specific antisera showed RF activity. We could therefore not exclude the possibility that the reaction was not with RF-positive proteins but with molecules binding such proteins.

Both V_K and V_H subgroup restrictions have been demonstrated for IgM RF [6, 25]. Førre *et al.* have demonstrated shared idiotypic antigens between certain IgG and IgM RF, but no V_H subgroup restriction among these proteins [5]. Our fractions that contained the common antigenic determinants contained IgG and IgM. The relationship between these antigenic determinants and those found in RF by Førre *et al.* [5] remains to be investigated. No information is given about whether their anti-idiotypic antisera reacted with all molecules with RF activity.

The IC sera contained unique immunoglobulins with common antigenic determinants. These common antigenic determinants occurred both in 7S components and in IC and RC and could in one case be demonstrated in the Fab γ part of the 7S molecules with affinity for the specific antisera. Work is under way to relate the common antigenic determinants to a certain part of the reactive IC and RC molecules.

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CIRCULAR DICHROISM OF IMMUNE COMPLEXES WITH RHEUMATOID FACTOR ACTIVITY

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Abstract—The circular dichroism (CD) of IgG complexes with RF activity and that of monomeric IgG without this activity, and isolated from the same individual, were compared. The IgG complexes had a significantly deviant CD spectrum in the near u.v. region, both before and after dissociation, whereas the monomeric IgG had a normal spectrum.

Immunoglobulins were isolated from the same serum with the use of a specific antiserum against unique determinants in some IgG complexes with RF activity. Both before and after dissociation the CD spectrum in the far u.v. region of these immunoglobulins differed significantly from that of the above-mentioned preparations.

The results confirmed that the structure of IgG in the RF-active complexes differed from that of normal IgG. The immunoglobulins with the unique determinants had, in turn, a structure that was not found in the pool of the RF-active IgG molecules or in normal IgG.

INTRODUCTION

IgG-complexes occur in the sera from individuals with rheumatoid arthritis (RA) (Kunkel *et al.*, 1961; Pope *et al.*, 1975; Roberts-Thomson *et al.*, 1976) and play a major role in the immunopathology of the disease (Ziff, 1973; Zvaifler, 1974). Previously, we isolated IgG complexes with rheumatoid factor (RF) activity from RA sera and found that the IgG in these complexes differed from normal IgG, for example, concerning subclass distribution and content of neuraminic acid (Hansson *et al.*, 1981). Circular dichroism (CD) might be useful for detecting conformational differences between immunoglobulins. Here, the question was whether CD could be used to detect conformational differences between the IgG in the previously mentioned complexes and normal IgG.

The immunogens that elicit the formation of RF *in vivo* are still unknown. Specific rabbit antibodies prepared against unique antigenic determinants in the previously mentioned IgG complexes with RF activity also reacted with some, but not with all, RF-active IgG complexes in other RA sera (Hansson *et al.*, 1981). The question was whether CD could be used to confirm the structural differences between the molecules with the unique determinants and the pool of IgG complexes.

In this investigation, the CD was measured for

IgG complexes with RF activity, for monomeric IgG without RF activity and for immunoglobulins with the unique antigenic determinants, all isolated from one RA serum. The result showed significant differences not only between IgG in the IgG complexes and the monomeric IgG, but also between the immunoglobulins with the unique antigenic determinants and the fraction containing the pool of IgG complexes.

MATERIALS AND METHODS

Serum

The serum (OS) was from a patient with sero-positive rheumatoid arthritis (RA) and had been stored at -20°C . It contained 27% IgG complexes ($\geq 9\text{S}$, $< 19\text{S}$).

Isolation of IgG complexes and monomeric IgG

The IgG complexes had RF activity and were isolated by affinity chromatography to immobilized heat-denatured human IgG as described earlier (Hansson *et al.*, 1981). The IgG complexes and the monomeric IgG without affinity to heat-denatured human IgG were further purified by ion-exchange chromatography (DEAE-Sephacel; 0.02 M sodium phosphate, pH 8.0). Monomeric reference IgG was isolated from a commercial IgG preparation for human use (Gamastan, Cutter Laboratories,

Berkeley, California, U.S.A.) by gel filtration (Sephacryl S-300; 0.05 *M* sodium phosphate, 0.5 *M* NaCl, pH 7.0). Immunoglobulins with unique antigenic determinants (Hansson *et al.*, 1981) were isolated by affinity chromatography to immobilized F(ab')₂γ of specific rabbit antibodies against IgG with RF activity isolated from a homologous serum (BL; Hansson *et al.*, 1981). The *s*-rate distribution of the components in the samples was determined as described before (Hansson *et al.*, 1981).

CD

CD spectra were obtained with a Jasco J-40 A spectropolarimeter at 22°C. The cell length was 1.00 cm between 250 and 320 nm and 0.10 cm below 250 nm. Three solvents were used: 0.012 *M* sodium phosphate, 0.014 *M* NaCl, pH 7.3; 0.004 *M* sodium acetate, pH 5.4; and 0.05 *M* sodium phosphate, pH 11.0. The samples were filtered before CD analysis through sterile 0.45-μm cellulose acetate membranes. The light absorbancy at 280 nm ($E_{1\%}^{1\text{cm}}$ 280 nm, = 12.5; Metzger, 1970) was below 1.5 absorbancy units. Furthermore, the protein concentration was determined immediately before CD analysis by the Folin phenol method (Lowry *et al.*, 1951). The mean residue ellipticity (degrees · cm² · dmole⁻¹) was calculated as $[\theta]_{\lambda} = \frac{\theta \cdot M_0}{c \cdot l}$, where *c* is the protein concentration (g/100 ml); *l* the pathlength in decimeters; *M*₀ the mean residue weight taken as 110, and θ the recorded ellipticity in degrees. No Lorentz corrections were made. The errors due to noise were ± 1 degree · cm² · dmole⁻¹ above 250 nm and ± 100 degrees · cm² · dmole⁻¹ at 218 nm. In most cases two separate preparations of each sample were analysed in duplicate. The spectrum that is presented for each sample is the mean value of all the recorded spectra of the sample.

Conclusions about differences between CD spectra were based on either of two calculated degrees of significance:

- (1) The significance was calculated with *t*-statistics (*P* = 0.01) of the majority of the samples.
- (2) The significance was calculated as 2 SD for the CD spectra obtained for IgG complexes, monomeric IgG and reference IgG at pH 11.0, in which cases only one preparation was analysed in duplicate.

The mean residue ellipticities for monomeric (7S) IgM prepared from a macroglobulinaemic IgM (κ) were at pH 7.3, 218 nm: -3165; and at pH 11.0, 218 nm: -3341; 254 nm: +128.7.

The mean residue ellipticities for IgA were at pH 7.3, 218 nm: -3900 (Underdown & Dorrington, 1974) and this value was also used at 218 nm at pH 11.0. At 254 nm at pH 11.0 the value of reference IgG was used.

The mean residue ellipticity of the mixture of IgG (or IgG complexes) and IgM and IgA were calculated from the previously mentioned data, according to Dorrington & Smith (1972). The standard deviations given for the mean residue ellipticity of the mixtures in Table 1 are those obtained experimentally for reference IgG and IgG complexes, respectively.

Protein determinations

Human serum proteins were identified by immunoelectrophoresis (Sheiddegger, 1955) and double immunodiffusion (Ouchterlony, 1962) with monospecific rabbit antisera. IgA was quantified with the use of laser turbidimetry (Blom & Hjörne, 1976) and IgM by single immunodiffusion (Hansson *et al.*, 1981).

RESULTS

The CD (195–320 nm) of IgG with RF activity (90% IgG complexes and 10% monomeric IgG) was compared with the CD of monomeric IgG without RF activity from the same patient and both were compared with a reference IgG isolated from a commercial IgG preparation. The IgG samples used were immunologically

Table 1. Comparison of the mean residue ellipticities ± 1 SD (degrees · cm² · dmole⁻¹) of the immunoglobulins with the unique determinants and two reference mixtures

Sample	218 nm		254 nm
	pH 7.3	pH 11.0	pH 11.0
Preparation with unique determinants	-3882 ± 377	-3869 ± 256	97.2 ± 4.0
Normal IgG reference mixture ^a	-2905 ± 242	-3042 ± 64	59.8 ± 1.6
IgG complex reference mixture ^b	-2829 ± 137	-3042 ± 33	54.3 ± 1.6

The reference CD spectra were calculated as follows:

^aNormal IgG reference mixture: $0.6 [\theta]_{\lambda}^{\text{normal IgG}} + 0.3 [\theta]_{\lambda}^{\text{IgM}} + 0.1 [\theta]_{\lambda}^{\text{IgA}}$.

^bIgG complex reference mixture: $0.6 [\theta]_{\lambda}^{\text{IgG complexes}} + 0.3 [\theta]_{\lambda}^{\text{IgM}} + 0.1 [\theta]_{\lambda}^{\text{IgA}}$.

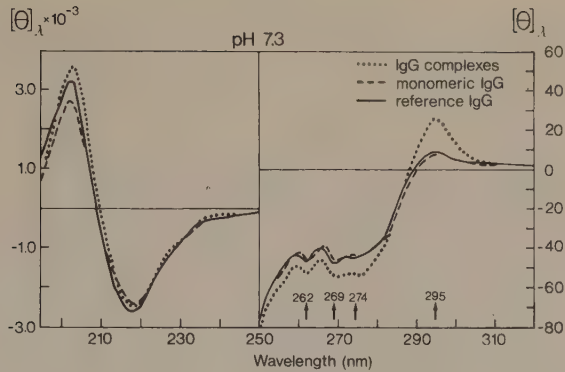


Fig. 1. CD, at pH 7.3, of IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The arrows indicate significant differences between the IgG complexes and the reference IgG. The standard deviations were $137\text{--}242$ degrees $\cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ at 218 nm and $0.6\text{--}3.5$ (median value 1.7) degrees $\cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ above 250 nm .

pure and contained polyclonal IgG with kappa and lambda light chains. The CD spectrum of the non-dissociated IgG complexes at pH 7.3 was quantitatively different from the corresponding CD spectra, which were identical, of the other two IgG preparations at all transitions in the near u.v. region (Fig. 1). The CD spectra obtained at pH 5.4 (Fig. 2), when the IgG complexes had dissociated, were similar to those obtained at pH 7.3, but the positive ellipticity at 295 nm was less for the dissociated complexes than for non-dissociated complexes (compare Figs 1 and 2).

Ultracentrifugal analysis showed that the IgG complexes also dissociated at pH 11.0. The CD spectra of the three IgG preparations at this pH (Fig. 3) differed qualitatively and quantitatively from those obtained at pH 7.3. It was of interest

to note that now the reference IgG had mean residue ellipticities between those of IgG from dissociated complexes and native monomeric IgG, which together constituted the IgG pool of the RA serum.

One immunoglobulin preparation containing unique determinants was isolated by affinity chromatography of the RA serum to the immobilized $\text{F(ab')}_2\gamma$ of a specific rabbit antiserum against IgG complexes with RF activity that had been isolated from a homologous serum. This preparation consisted of 40% 7S and 60% complexes ($\geq 9\text{S}$, $< 19\text{S}$) and contained not only IgG, but also 30% IgM ($< 19\text{S}$), 10% IgA and less than 5% Clq. The CD obtained at pH 7.3 and 11.0 was compared with the CD of two reference mixtures at the same pH (Table 1). The reference mixtures were normal

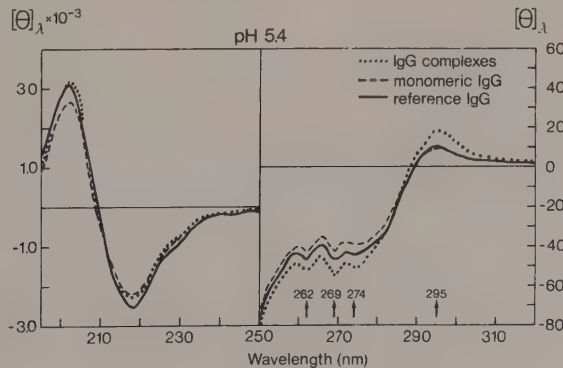


Fig. 2. CD, at pH 5.4, of dissociated IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The arrows indicate significant differences between the dissociated IgG complexes and the reference IgG. The standard deviations were $33\text{--}192$ degrees $\cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ at 218 nm and $0.4\text{--}1.6$ (median value 1.0) degrees $\cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ above 250 nm .

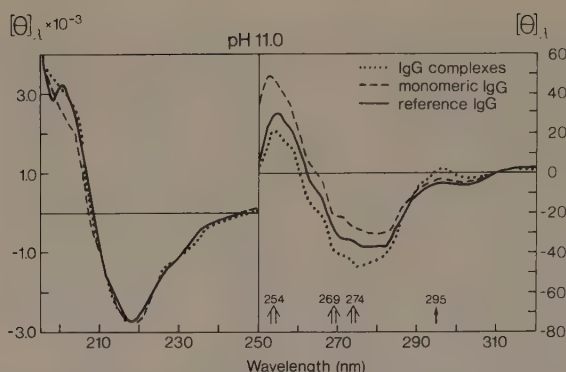


Fig. 3. CD, at pH 11.0, of dissociated IgG complexes with RF activity and monomeric IgG without RF activity and isolated from one RA serum compared with a reference IgG. The solid arrow indicates a significant difference between the dissociated IgG complexes and the reference IgG. The blank arrows indicate significant differences between the dissociated IgG complexes, the monomeric IgG and the reference IgG. The standard deviations were 33–75 degrees $\cdot$ cm² $\cdot$ dmole⁻¹ at 218 nm and 0.1–4.5 (median value 0.8) degrees $\cdot$ cm² $\cdot$ dmole⁻¹ above 250 nm.

IgG and IgG complexes, respectively, and 7S IgM and IgA, in the same proportions as in the preparation. At pH 7.3 the CD of the preparation differed significantly from that of the two reference mixtures at 218 nm. At pH 11.0, that is, when the complexes had dissociated, the ellipticity of the preparation at 218 nm was still significantly lower than those of the two reference mixtures, which were similar. At pH 11.0, however, differences in ellipticity also occurred at 254 nm. Thus, the structure of the components with unique determinants reflected by the ellipticity at 218 nm at a neutral and an alkaline pH and at 254 nm at an alkaline pH, differed from that of the immunoglobulins in the two reference mixtures.

DISCUSSION

The agreement between our results obtained with the reference IgG and those obtained with IgG by others (Doi & Jirgensons, 1970; Dorrington & Smith, 1972) at different pH levels accentuated the significance of the differences between our reference IgG and the IgG preparations from the RA serum.

Differences between the IgG complexes and the reference IgG were obtained at several transitions in the near u.v. region. The transition at 295 nm has been attributed to tryptophan and the other transitions in the near u.v. region more generally to aromatic amino acids and disulphide bonds in asymmetric environments (Dorrington & Smith, 1972). Thus, tryptophan, other aromatic amino acids and disulphide bonds in asymmetric

environments contributed to the abnormal structure of IgG in the complexes. The absorption band at 295 nm was reduced when the IgG complexes had dissociated and reflected mainly the optical activity of tryptophan.

Small differences have been reported in the CD spectra of IgG isolated from RA serum and normal IgG (Johnson *et al.*, 1974) at 280 nm at neutral pH but the correlation of these differences with RF activity are still obscure. In our study, the IgG complexes with RF activity isolated from RA serum showed quantitative differences at several transitions in the near U.V. region compared to the reference IgG, whereas IgG without RF activity isolated from RA serum had a normal CD spectrum at a neutral pH. Some IgG complexes had not been formed due to self-association of RF (Hansson *et al.*, 1981). It therefore remains to be investigated whether the differences in CD spectra are related to the structure of IgG with or without RF activity.

The ellipticity at 218 nm of the immunoglobulins with common antigenic determinants was 20% more negative than that of the corresponding mixtures of normal IgG (or IgG complexes), IgM and IgA. Negative ellipticity values at 218 nm, deviating as much as 30% from the value of normal rabbit IgG have been reported for individual specifically purified rabbit antibodies (Bear *et al.*, 1974). The components with unique antigenic determinants might also comprise a population with special antibody properties since the specific antiserum reacted with the F(ab')₂ part of the components (Hansson *et al.*, 1981).

The unique antigenic determinants were not related to RF and were only exposed in a minor part of the immunoglobulin complexes with RF activity (Hansson *et al.*, 1981). This was in agreement with the difference between the CD of the components with the unique antigenic determinants and that of the IgG complexes with RF activity from the same patient. It would appear that the unique antigenic determinants were related to immunoglobulins with an abnormal conformation. Whether this conformation was present in unique RF or in immunoglobulins bound to RF remains to be investigated.

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Factors affecting IgA related hyperviscosity

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SUMMARY

Sera from 14 patients with an IgA M-component, six of whom had myelomatosis and eight with benign monoclonal gammopathy (BMG) were analysed. All six sera from patients with high IgA (>40 g/l) and total protein (>100 g/l) concentrations were hyperviscous (HV). Four of these six patients also had hyperviscosity syndrome (HVS). There was no correlation between the quantity of IgA dimers or polymers and the presentation of HV and HVS. The binding between IgA and albumin and α_1 -anti-trypsin was not covalent. Differences in the microenvironment of S–S bonds or of aromatic amino acids between isolated monoclonal monomeric and dimeric IgA were demonstrated with circular dichroism. Besides that, differences in hydrophobicity (exposure of aromatic amino acids) between IgA from normal serum and monomeric and dimeric IgA from a myeloma serum were revealed using hydrophobic interaction chromatography. The significance of hydrophobic interactions involving IgA and the influence of such forces on the circulation of the molecules are discussed.

INTRODUCTION

Hyperviscosity (HV) of the serum is relatively common in patients with IgA myeloma (Mestecky *et al.*, 1977; Preston *et al.*, 1978; Roberts-Thomson, Mason & MacLennan, 1976; Dine, Guay & Snyder, 1972; Sugai, 1972; Virella, Preto & Graca, 1975; Tuddenham *et al.*, 1974; Freil, Maldonado & Gleich, 1972) and may cause a hyperviscosity syndrome (HVS).

HV has been correlated with the high serum concentration of the paraprotein (Mestecky *et al.*, 1977; Preston *et al.*, 1978; Roberts-Thomson *et al.*, 1976; Dine *et al.*, 1972; Sugai, 1972; Virella *et al.*, 1975; Tuddenham *et al.*, 1974; Freil *et al.*, 1972), with the high serum content of IgA polymers (Preston *et al.*, 1978; Roberts-Thomson *et al.*, 1976; Virella *et al.*, 1975; Tuddenham *et al.*, 1974) and with interactions between IgA and other serum proteins such as albumin and α_1 -anti-trypsin (Mestecky *et al.*, 1977; Dine *et al.*, 1972; Virella *et al.*, 1975; Tomasi & Grey, 1972). The relationship between HV and the above parameters is still unclear, as well as the relation between HV and HVS. This work was performed to scrutinize these relationships.

MATERIALS AND METHODS

Clinical material. The material consisted of 15 patients seen at the Department of Internal Medicine, University Hospital, Linköping, during a 5 year period (1975–1980). Of the 14 cases with a monoclonal IgA increase, six were classified as multiple myeloma because of radiographic

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multiple lytic bone lesions and eight as benign monoclonal gammopathy (BMG) (Table 1). Two BMG cases required cytostatic treatment because of HVS or increasing IgA levels. The patient with a polyclonal IgA increase had coeliac disease. Normal human serum was a pool of serum samples from 50 blood donors.

Rabbit antisera against normal human serum proteins. Anti-IgA, -IgG and -IgM were prepared in our laboratory, by hyperimmunizing rabbits with the purified immunogen in Freund's complete adjuvant and were appropriately absorbed. Anti- α_1 -anti-trypsin was obtained from Seward Laboratory, London, UK; anti-albumin from DAKO-immunoglobulins Ltd, Copenhagen, Denmark; anti- κ and - λ light chains from Behringwerke AG, Marburg, West Germany and anti-IgA1 and -IgA2 from Nordic Immunological Laboratories, Tilburg, The Netherlands.

Isolation of myeloma IgA. IgA was isolated from serum by preparative agarose gel electrophoresis. 7S and 9S IgA in the IgA sample were separated by repeated gel filtration on Sephacryl S-300 (Pharmacia, Sweden). Analytical ultracentrifugation was used to control the content of 7S and 9S component in the isolated preparations. Double immunodiffusion and immunoelectrophoresis against appropriate antisera were used to check the purity of the preparations.

Electrophoresis. Agarose gel electrophoresis and immunoelectrophoresis (Scheidegger, 1955) were run in 0.8% agarose gel, 0.075 M barbital buffer, 2 mM calcium lactate, pH 8.6.

Protein quantitation. Serum total protein was determined according to Weichselbaum (1946) (normal range; 70–80 g/l). IgG (normal range; 7.0–14.0 g/l) and IgA (normal range; 1.2–3.5 g/l) were measured with a nephelometric technique (Lizana & Hellsing, 1974), IgM (normal range; 0.5–1.8 g/l) was measured with Mancini radial immunodiffusion technique (Mancini, Carbonara & Heremans, 1965). Before and after reduction of the samples α_1 -anti-trypsin was determined with radial immunodiffusion (Mancini *et al.*, 1965). Albumin was quantified according to Doumas, Watson & Biggs (1971).

Plasma viscosity. This was measured at 37°C, with a Wells-Brook-field microviscometer (Wells, Denton & Merrill, 1961) (normal range; $\eta_{rel} = 1.2$ – 1.6).

Dissociation of protein complexes. One or another of the following three solvents was used: (1) acid medium; 0.1 M glycine-HCl, pH 3.0; (2) alkaline medium; 0.075 M Tris, 0.2 M 2-aminobutane, 10^{-3} M CaCl_2 , pH 11.0; (3) reduction; 0.1 M mercaptoethanol at 37°C overnight.

Table 1.

Patient	Type of myeloma*	Plasma viscosity	Hyperviscosity syndrome
1	MM	3.1	tiredness, epistaxis, somnolence
2	MM	2.8	—
3	MM	2.7	—
4	MM	2.6	tiredness, stupor, gastrointestinal bleeding
5	MM	1.7	—
6	MM	1.4	—
7	BMG	3.3	vertigo, tiredness, impaired vision
8	BMG	2.3	vertigo, nausea, impaired vision
9	BMG	1.6	—
10	BMG	1.5	—
11	BMG	1.4	—
12	BMG	1.3	—
13	BMG	1.3	—
14	BMG	1.3	—
15	poly	1.5	—

* MM = multiple myeloma; BMG = benign monoclonal gammopathy; Poly = polyclonal IgA increase.

Ultracentrifugal analysis. This was performed with an An-D rotor in a Beckman analytical ultracentrifuge Model E with Schlieren Optics as described before (Hansson, Uesson & Alkner, 1981).

Circular dichroism (CD) analyses. These were performed as described elsewhere (Alkner *et al.*, 1982). Two solvents were used for each sample: 0.012 M sodium phosphate, 0.015 M NaCl, pH 7.3 and 0.05 M sodium phosphate, pH 11.0. The protein concentration was determined with the Folin phenol method (Lowry *et al.*, 1951) immediately before CD analysis. The mean residual ellipticity ($\text{degrees.cm}^2.\text{dmole}^{-1}$) was calculated as $\{\theta\}_x = (\theta.M_0)/(c.l)$ (c = the protein concentration in g/100 ml; l = the cell length in dm; M_0 , the mean residue weight = 110; θ = the reduced ellipticity in degrees). No Lorenz corrections were made. The errors due to noise were $\pm 1 \text{ degree.cm}^2.\text{dmole}^{-1}$ above 250 nm and $\pm 100 \text{ degrees.cm}^2.\text{dmole}^{-1}$ at 217 nm. Every sample was analysed in duplicate. The significance of differences between spectra was calculated as 2 s.d.

Hydrophobic interaction chromatography. This was performed on phenyl-Sephacrose CL-4B (Pharmacia, Sweden) according to Doellgast & Plaut (1976). The serum samples (normal human serum and serum from patient 4) were defatted according to Burstein & Samaille (1959) and then dialysed first against saline containing 2 g/l EDTA and second against the start buffer. Stepwise elution was performed with (a) 1M $(\text{NH}_4)_2\text{SO}_4$, 0.04 M Tris-acetate, pH 7.6; (b) 0.8 M $(\text{NH}_4)_2\text{SO}_4$, 0.032 M Tris-acetate, pH 7.6; (c) 0.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.024 M Tris-acetate, pH 7.6; (d) 0.4 M $(\text{NH}_4)_2\text{SO}_4$, 0.016 M Tris-acetate, pH 7.6; (e) 0.25 M NaCl, 0.05 M Tris-acetate, pH 8.0 and (f) 0.25 M Tris, pH 10.5.

RESULTS

Six of the 14 patients with IgA M-components showed elevated plasma viscosity and four of them also showed HVS (Table 1).

All sera from the six patients with HV contained more than 100 g/l of total serum protein and more than 40 g/l of IgA (Fig. 1). The IgG and IgM content was normal or decreased in all sera.

Analysis showed that 10 of the sera contained 9S complexes in concentration of 2–56 g/l (Fig. 2). The remaining four contained lower amounts. Complexes (3 g/l) with a sedimentation rate of 13S were detected only in the serum from patient 3. Immunoelectrophoresis and double immunodiffusion against appropriate antisera showed that 90% or more of the isolated complexes from sera 1, 4 and 8 consisted of IgA. The serum from patient 8 was exceptional in that it contained a very large total amount of IgA (56 g/l), but only 2% IgA complexes (9S), and this ratio remained constant during a period of 3 years. The development of HVS was not related to the content of IgA complexes (Fig. 2).

Immunoelectrophoresis indicated that all sera except two (patients 10 and 15) contained IgA-albumin complexes and all but three (patients 10, 11 and 15) contained IgA- α_1 -anti-trypsin complexes. Analyses of all sera in dilutions from 1:1 to 1:50 showed that the amount of such complexes was high in the sera with a high IgA concentration (patients 1–4, 7 and 8), and lower in the others. The total amount of α_1 -anti-trypsin and albumin in the sera was normal or low. Both types of complexes dissociated in acid medium (non-covalent binding) as well as on reduction with mercaptoethanol (covalent binding). The IgA dimers dissociated on reduction with mercaptoethanol, but not when dissolved in an acid medium. This indicated that the major complexation was between IgA dimers (covalently bound), which were non-covalently bound to albumin and α_1 -anti-trypsin, respectively.

The conformation and hydrophobicity of a monoclonal IgA isolated from a HVS patient (serum 4) was studied. Circular dichroism (CD) spectra were determined for the isolated 7S and 9S monoclonal IgA (Fig. 3). No significant differences were detected at neutral pH. However, at pH 11 significant differences at 251 nm were obtained (Fig. 3). 7S IgA gave a higher positive ellipticity than 9S IgA. The conformational differences between the two myeloma proteins from the same individual were thus either related to the exposure of aromatic amino acids in the molecules (hydrophobicity) or to the microenvironment of S–S bonds.

The hydrophobicity of the 7S and 9S IgA in serum 4 was compared with that of 7S IgA in normal serum by hydrophobic interaction chromatography on phenyl-Sephacrose CL-4B. The IgA in

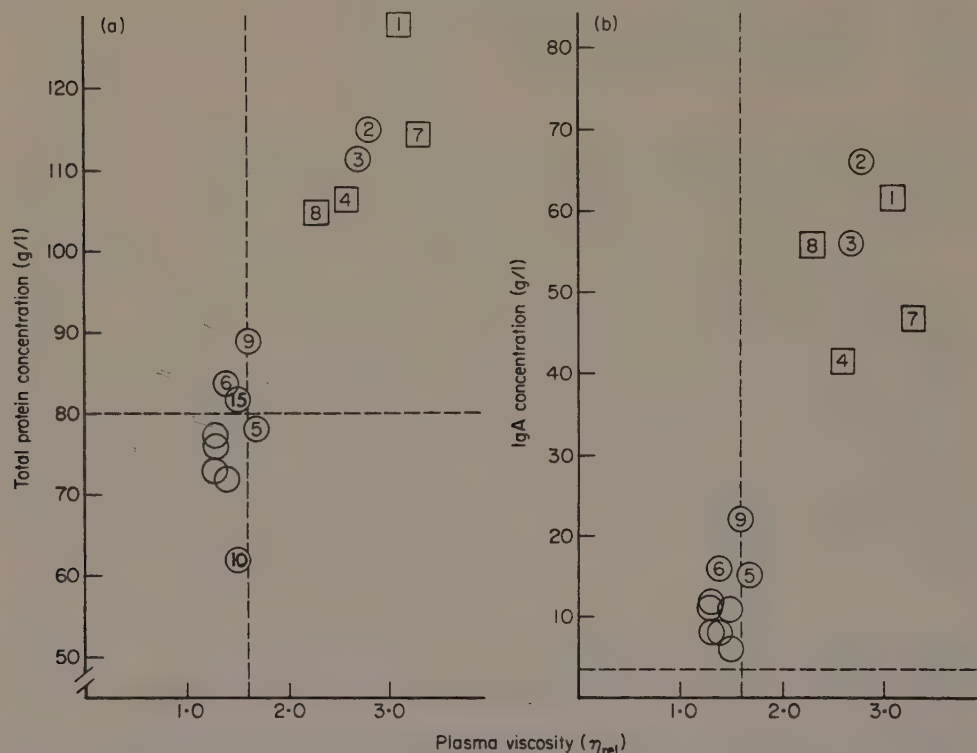


Fig. 1. Total protein concentration (a) and IgA concentration (b) in the 15 samples plotted against the plasma viscosity. The dotted lines denote the upper limits of the normal ranges. The squares represent patients with HVS and the numerals within the symbols, the patients' numbers.

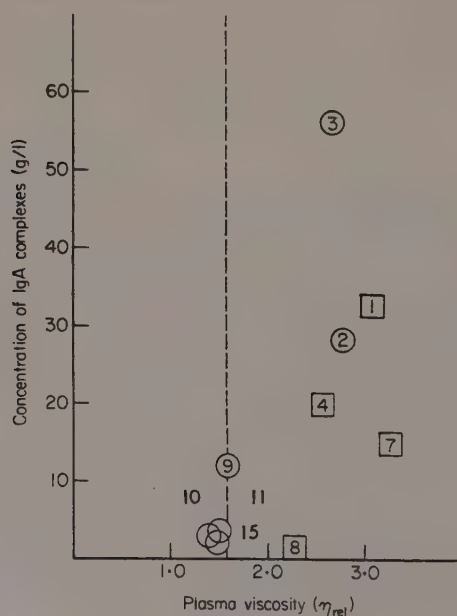


Fig. 2. Concentration of IgA complexes (9S) in the sera plotted against the plasma viscosity. The dotted line denotes the upper limit of the normal viscosity range. The squares represents patients with HVS and the numerals within the symbols, the patients' numbers.

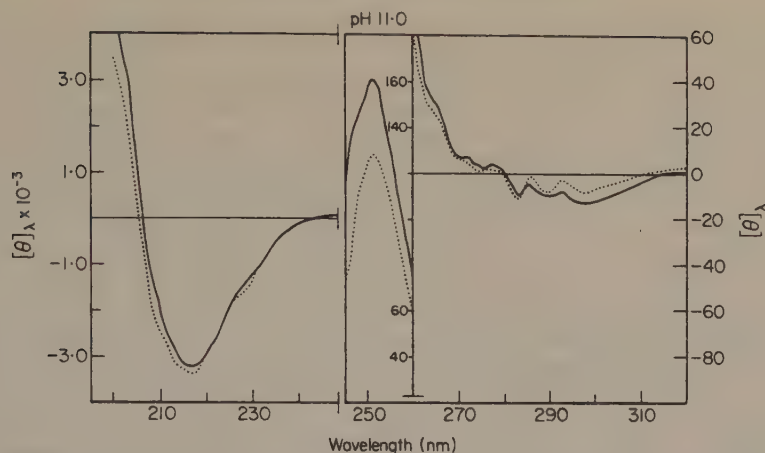


Fig. 3. CD, at pH 11.0, of 7S IgA (—) and 9S (....) IgA isolated from patient 4. The standard deviations were $57\text{--}251 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ at 217 nm and $0.1\text{--}3.8$ (median value 0.3) $\text{degrees} \cdot \text{cm}^2 \cdot \text{dmole}^{-1}$ above 250 nm.

serum 4 had a higher affinity to phenyl-Sephadex than had the IgA in normal serum, which was eluted with the solvents containing ammonium sulphate (see Materials and Methods). Two IgA fractions were obtained from serum 4. The composition of these was determined by immunodiffusion analysis against appropriate antisera and analytical ultracentrifugation. Ninety per cent of the 7S IgA was eluted at pH 8 while 90% of the 9S IgA was eluted at pH 10.5. This meant that both the 7S and 9S IgA in serum 4 was more hydrophobic than normal IgA. The 9S IgA was in turn more hydrophobic than the 7S IgA.

The IgA paraprotein belonged in all cases to subgroup 1 and contained lambda light chains in six (patients 3, 4, 6, 8, 13 and 14) and kappa light chains in the others. The conformations of IgA that were related to IgA subclass or light chain group were not correlated with the hyperviscosity.

DISCUSSION

All the sera with a high IgA concentration ($> 40 \text{ g/l}$) were hyperviscous, whether they originated from patients with benign monoclonal gammopathy (BMG) or with myelomatosis. Our results indicated that the reported rare occurrence of HVS in BMG (Leading article, 1971) can be explained by the lower IgA concentrations in most BMG than in myelomatosis. Our data agree with previous findings (Lindström & Dahlström, 1978) that the differences between the two conditions are quantitative rather than qualitative. The intrinsic viscosity of IgA monomers and dimers is normally similar (Preston *et al.*, 1978). The viscosity of serum should therefore depend more on the total amount of IgA than on the ratio between monomeric and dimeric IgA, which agrees with our observation that sera with a similar viscosity and IgA content showed up to 20-fold differences in dimer concentrations.

Most of the sera studied here like those studied by others (Mestecky *et al.*, 1977; Roberts-Thomson *et al.*, 1976; Dine *et al.*, 1972; Virella *et al.*, 1975; Tomasi & Grey, 1972) contained IgA-albumin and IgA- α_1 -anti-trypsin complexes.

Our experiments showed that IgA dissociated from albumin and α_1 -anti-trypsin in an acidic medium. This observation is in opposition to previous report (Tomasi & Grey, 1972) of covalent binding between these proteins. Albumin contains hydrophobic surface areas involved in the binding of aromatic substances (Peters, Hiroshi & Anfinsen, 1973), fatty acids (Hofstee & Otilio, 1978) and other aliphatic compounds (Peters *et al.*, 1973). This applies also to parts of the immunoglobulin molecules (Peters *et al.*, 1973; Hofstee & Otilio, 1978; Hofstee, 1979). There is also evidence that hydrophobicity is a major factor in stabilizing protein-protein interactions (Chothia

& Janin, 1975). It was therefore reasonable to assume that the IgA-albumin and IgA- α_1 -anti-trypsin complexes were formed partly by hydrophobic interactions between hydrophobic regions within the molecules. The existence of these complexes demonstrated the tendency for IgA to form hydrophobic interactions. This was confirmed when we showed that IgA in the HV serum was more hydrophobic than was normal IgA. This increased hydrophobicity of IgA can be of importance for the transport of aromatic and aliphatic compounds in the circulation. The passage of dimeric IgA from blood to liver and bile requires its binding with secretory component in the hepatocyte membranes (Hall & Andrew, 1980). A blocking of these binding sites hinders the circulation of IgA (Hall & Andrew, 1980). Further research will unravel whether the binding of hydrophobic substances will hinder the circulation of IgA or will lead to that IgA carries hydrophobic molecules from the blood into the liver as it has been shown to carry IgG (Peppard *et al.*, 1981).

The CD spectra of the monoclonal monomeric and dimeric IgA were different at 251 nm when dissolved in an alkaline medium. This indicated differences in the microenvironment of S-S bonds or of aromatic amino acids (Jirgensons, 1973). Similar differences in the CD spectra were observed for monomeric and dimeric IgG (Alkner *et al.*, 1982). The IgG dimers contained no intermolecular disulphide bonds (Alkner *et al.*, 1982). Our conclusion was that the observed differences in the CD spectra for IgA were mainly related to the microenvironment of aromatic amino acids. This conclusion was compatible both with a hydrophobic bond between IgA and albumin and with the observation that monomeric and dimeric IgA had different hydrophobicities. Hydrophobic affinity chromatography also showed that dimeric IgA had the greatest propensity for hydrophobic binding, a tendency which might explain why IgA-albumin and IgA- α_1 -anti-trypsin complexes dissociated on reduction with mercaptoethanol, that is, as a result of the dissociation of IgA dimers into monomers.

Our results indicated that further research into the relationship between plasma hyperviscosity and HVS should concentrate on studies of hydrophobicity and the involved conformational changes of IgA. These parameters may well be of fundamental importance with respect to the blood-liver circulation of IgA.

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